Tissue engineering of skeletal muscle

Koning, Merel

TISSUE ENGINEERING OF SKELETAL MUSCLE

FOR PATIENTS WITH FACIAL PARALYSIS

Merel Koning

Financial support for the publication of this thesis is gratefully acknowledged:
University of Groningen, Groningen Graduate School of Medical Sciences, Afdeling Plastische Chirugie UMCG, Nederlandse Vereniging voor Plastische Chirurgie, Stichting Wetenschappelijk Onderzoek Assistenten Heelkundige Specialismen, Stichting Kortjakje, Van Wijngaarden Medical, Nederlandse Vereniging voor Biomaterialen en Tissue Engineering.

Koning, Merel
Tissue engineering of skeletal muscle for patients with facial paralysis
Proefschrift Groningen

ISBN: 978-90-367-5560-3
ISBN: 978-90-367-5561-0 / electronische versie

Cover: www.studioivo.nl
Lay-out: M. Koning
Printed by BOXPress Publishers, Oisterwijk

RIJKSUNIVERSITEIT GRONINGEN

TISSUE ENGINEERING OF SKELETAL MUSCLE

for patients with facial paralysis

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
woensdag 13 juni 2012
om 16.15 uur

door

Merel Koning
geboren op 15 november 1982
te Den Helder

Promotores:	Prof. dr. P.M.N. Werker
	Prof. dr. M.J.A. van Luyn
Copromotor:	Dr. M.C. Harmsen
Beoordelingscommissie:	Prof. dr. W.F.J. Feitz
	Prof. dr. B. van der Lei
	Prof. dr. M.J. Post

Paranimfen: Miriam Boersema
Guido H.C.G. Dolmans

CONTENTS

CHAPTER 1

INTRODUCTION AND OUTLINE OF THIS THSIS

INTRODUCTION

The human face holds 47 muscles that are responsible for facial expression [1]. Paralysis of facial muscles impairs facial expression, which causes significant physical, psychological and social distress.

Facial paralysis has different etiologies which are of congenital or of acquired nature. If an acquired facial paralysis is caused by for instance a trauma or idiopathic, the paralysis might be either self-limiting or reversible through surgery. However, if the paralysis persists for more than 18 months or has a congenital or degenerative cause, the paralysis is irreversible. For these patients with longstanding facial paralysis, reconstructive surgery, including static or dynamic reconstructions and additional intensive conservative, non-surgical therapies are warranted. Currently, autologous free flap transplantation offers the best chance to reconstruct a spontaneous smile [2,3] or synchronous eye closure [4]. However, downsides of current free flap reconstruction are that only one or two of the missing muscles can be replaced, and a completely symmetrical spontaneous smile or eye closure is seldom obtained. Besides, there might be undue swelling of the cheek or eyelids and patients are often hindered by the autostatic syndrome, a situation in which the muscle that elevates the corner of mouth during smiling also contracts when the patient attempts to wrinkle the lips [5], such as in kissing. Despite advancing knowledge and improved surgical techniques, these conventional treatment strategies improve the functionality of one or two facial muscles only partly, while genuine regeneration of the muscles and movement is not achieved [6,7].

Innovative strategies based on regenerative medicine, in particular tissue engineering of skeletal muscle, will yield new treatment modalities for patients with longstanding facial paralysis. Engineered muscle tissue can be customized to the individual patient. Therefore, it can restore facial expression. Thus, it can improve physical and psychological symptoms.

In tissue engineering, basically tissues are reconstructed using cells and scaffolds. Frequently, stem cells, *i.e.* progenitor cells are used, because these harbor a high differentiation capacity. A major challenge in tissue engineering is to dissect and master the regulatory mechanisms that govern cellular differentiation. As a progenitor cell for tissue engineering skeletal muscle, satellite cells seem ideal because they have the capacity to differentiate into skeletal muscle. Satellite cells are a cell population present in skeletal muscle. They play a critical role in postnatal skeletal muscle

homeostasis, growth, repair and regeneration. As first shown by electron microscopy, satellite cells are mononucleated cells located between the sarcolemma of a muscle fiber and the basal lamina that surrounds that fiber [8]. This anatomical compartment serves as an instructive environment, *i.e.* a niche, in which satellite cells remain in a quiescent state or can be activated to divide and differentiate towards myoblasts and fuse into myotubes in response to stimuli associated with muscle growth, damage and repair. This makes satellite cells ideal for tissue engineering skeletal muscle.

Tissue engineering is partly an *in vitro* process; therefore, the satellite cell niche needs to be recreated to support satellite cells and facilitate activation and differentiation. Hence, an instructive scaffold is necessary as a carrier to provide structural support and participate in the regulation of cellular function. Then, for survival *in vivo*, tissue engineered skeletal muscle requires adequate vascularisation for efficient transport of oxygen, carbon dioxide, nutrients and waste products. And finally, innervation should be established. Facial muscle repair may be feasible by tissue engineering because these muscles are relatively small and thin, and are required to produce relative limited forces compared to most other skeletal muscles. For innervations, *i.e.* to replace the lost nerve, most patients still have a healthy facial nerve on the contra-lateral facial side. In addition, there are otherwise suitable options for innervation from other cranial nerves such as the hypoglossal and masseteric nerve.

OUTLINE OF THIS THESIS

The main objective of this thesis is to improve tissue engineering of human skeletal muscle by providing new fundamental insights and methods. Therefore, an extensive overview of the state of play in the field of tissue engineering of skeletal muscle is given in **chapter 2**. We discuss characteristics of facial muscles and the prerequisites for tissue engineering of skeletal muscle including progenitor cells, scaffolds, and current strategies for myogenesis, vascularisation or innervation.

In this thesis, the focus is on dissecting regulatory mechanisms in human myogenic satellite cells during the initial stages of myogenesis. And furthermore on the assessment of muscle maturation of an engineered muscle construct *in vivo*.

In order to engineer human muscle tissue, human satellite cells need to proliferate *in vitro* to obtain sufficient cell numbers and further differentiate to form muscle tissue. Fundamental knowledge of the processes of proliferation and differentiation of human satellite cells is essential for tissue engineering of skeletal muscle. This offers

opportunities to alter or modify satellite cell differentiation during muscle tissue engineering. Thereby, we would be able to customize muscle tissue to the individual patients needs.

Besides the 'traditional' regulation of myogenesis by transcription factors, epigenetic regulators such as microRNAs have gained increased interest. MicroRNAs are small, non-coding RNAs, 20-22 nucleotides in length, involved in post-transcriptional gene regulation through inhibition of protein translation or enhancing messenger RNA degradation [9]. In **chapter 3** we discuss whether and if so, which microRNAs have a regulatory function in the differentiation of human satellite cells. By modifying human satellite cell differentiation, the engineering of muscle tissue could be improved. Therefore, in **chapter 4**, the application of this regulating function of microRNAs in tissue engineering skeletal muscle is investigated at 2D and 3D level.

In tissue engineering, the ultimate goal is *in vivo* application of engineered muscle tissue. At that point, human satellite cells will encounter hypoxic environments. In normal human muscle tissue, satellite cells respond to hypoxic muscle damage with activation, proliferation, differentiation, and maturation, while maintaining their reserve pool of satellite cells ref. Hypoxic environments might therefore even be beneficial in culturing satellite cells and further differentiation *in vivo*. Hence, a better understanding of the response of human satellite cells to changes in oxygen level is required. This is investigated and discussed in **chapter 5**.

As a proof of concept, *in vivo* maturation of an *in vitro* engineering 3D muscle construct is investigated in **chapter 6**. Although facial muscles are thinner than most other skeletal muscles, their thickness needs to exceed 0.2 mm to be functional, *i.e.* generate sufficient force to move facial skin. Vascularisation is important for the survival of satellite cells and myofibers after implantation. Therefore, we also investigated whether human endothelial cells are beneficial for the vascularisation of a tissue engineered muscle construct *in vivo*.

Finally, **chapter 7** discusses the main and important fundamental findings of this thesis and provides future perspectives of tissue engineering human skeletal muscle for the benefit of patients with longstanding facial paralysis.

CHAPTER 2

CURRENT OPPERTUNITIES AND CHALLENGES IN SKELETAL MUSCLE TISSUE ENGINEERING

Merel Koning, Martin C. Harmsen, Marja J.A. van Luyn, Paul M.N. Werker

J TISSUE ENG REGEN MED. 2009 AUG;3(6):407-15

ABSTRACT

The purpose of this article is to give a concise review of the current state of art in tissue engineering (TE) of skeletal muscle, the opportunities and challenges for future clinical applicability.

The endogenous progenitor cells of skeletal muscle *i.e.* satellite cells, show a high proneness to muscular differentiation, in particular exhibiting the same characteristics and function as its donor muscle. This suggests that it is important to use an appropriate progenitor cell, especially in TE facial muscles, which have a exceptional anatomical- and fibercomposition compared to other skeletal muscle.

Muscle TE requires an instructive scaffold for structural support and to regulate the proliferation and differentiation of muscle progenitor cells. Current literature suggests that optimal scaffolding could comprise of a fibrin gels and cultured monolayers of muscle satellite cells obtained through the cell sheet technique.

Tissue engineered muscle constructs require an adequate connection to the vascular system for efficient transport of oxygen, carbon dioxide, nutrients and waste products.

Finally, functional and clinically applicable muscle constructs depend on adequate neuromuscular junctions with neural cells. To reach this, it seems important to apply optimal electrical, chemotropic and mechanical stimulation during engineering, and discover other factors that influence its formation. Thus, in addition to approaches for myogenesis, we will discuss current status of strategies for angiogenesis and neurogenesis of TE muscle constructs, and the significance for future clinical use.

INTRODUCTION

Paralysis of facial muscles, which provide facial expression, may lead to great physical and social distress for the patient. Despite advancing knowledge and improved surgical techniques, outcome of current treatment remains at best suboptimal [10-12]. Tissue engineering (TE) may offer an alternative future solution. In general, TE employs progenitor cells and scaffolds that together generate the appropriate environment to functionally repair, replace or regenerate lost or damaged tissue. Engineered muscle tissue may be customized to the individual patient and offers an opportunity for patients to improve physical and psychological symptoms and reach near to normal mimical function.

Nevertheless, muscle TE has limitations and challenges. Specific myogenic progenitor cells, scaffolds to support growth and differentiation of progenitor cells, and bioactive factors are required. Functional skeletal muscle requires parallel alignment of muscle fibers with myosin/actin filaments, and acetylcholine receptors to create direct forces. Besides, the tissue engineered muscle must be vascularised and innervated for future clinical application. Furthermore, the scaffold must be biocompatible [13,14].

The purpose of this article is to give a concise review of the current state of art in TE of skeletal muscle and its significance for future clinical use.

FACIAL MUSCLE CHARACTERISTICS

The human face holds 23 paired facial muscles and one unpaired, the orbicularis oris muscle [15]. Unlike other skeletal muscles which are spanned out between bones, facial muscles attach at least on one side to skin. Contraction of the facial muscles gives facial expression by moving the skin.

Each facial muscle is made up out of 75 to 150 muscle fibers [16]. They are arranged into parallel bundles, which run from origin to insertion [17].

In general, two types of fiber can be distinguished: Type I, or slow twitch muscle fibers that produce large amounts of energy at a slow pace, which allows them to work for a long time without getting exhausted; Type II:, or fast twitch muscle fibers produce small amounts of energy very quickly. This enables rapid movements, but causing great fatigability. Type II fibers are further subdivided in type IIA and type IIB (Table 1).

Muscles are made up of a mixture of type I and type II muscle fibers. Muscles consisting mainly of type I fibers are red and well perfused, because they contain many capillaries. They depend on a rich supply of oxygenated blood. Type II fibers are primarily dependent on anaerobic metabolism, no rich blood supply is needed and consequently muscles that consist mainly of type II fibers are more white colored [18]. The zygomaticus minor has the largest proportion type II fiber (89.1 %) reported in human skeletal muscle. Besides, the zygomaticus major and minor muscles have a marked predominance of a subtype II fiber, intermediate between type IIA and type IIB. The presence of these oxidative type IIAB fibers indicates a high resistance to fatigue. Besides, facial muscles lack a firm insertion, static contraction and subsequent development of high tension is prevented. Therefore, blood circulation is not obstructed, and fatigability is further postponed [19].

Table 1. Characteristics of skeletal muscle fiber type.

	Type I	Type IIA	Type IIX	Type IIB
Contraction time	Slow	Moderately Fast	Fast	Very fast
Size of motor neuron	Small	Medium	Large	Very large
Resistance to fatigue	High	Fairly high	Intermediate	Low
Maximum duration of use	Hours	<30 minutes	<5 minutes	<1 minute
Force production	Low	Medium	High	Very high
Capillary density	High	Intermediate	Low	Low
Oxidative capacity	High	High	Intermediate	Low

PROGENITOR CELLS

An important prerequisite for engineering skeletal muscle is using a suitable, autologous progenitor cell. This cell should be cultured, expanded and capable to differentiate into the cell types of muscle tissue.

Mesenchymal stem cells from several different sources harbor myogenic potency.

Bone marrow-derived mesenchymal stem cells (MSCs) are able to differentiate into myoblasts with a high efficiency [20]. Myoblasts fuse with each other to form myotubes, which differentiate to muscle fibers. MSCs have a high proliferation capacity and are capable of self-renewal. This renders them suitable as progenitor cells for skeletal muscle TE. Furthermore, they can be transplanted autologous and expanded efficiently *in vitro* to reach therapeutic scale [20,21]. Expansion of harvested cells is necessary in TE to create sufficient volume of skeletal muscle in order to deliver enough force to be functional in future clinical application. However, myogenic lineage induction in MSCs is an additional differentiation step. Furthermore, harvesting MSCs from bone marrow is a relatively invasive procedure for the patient.
Skeletal muscles on the other hand, harbor their own endogenous organ-specific mesenchymal stem cells, *i.e.* satellite cells. These are, unlike MSCs, already prone to myogenic differentiation, and therefore seem more suitable for skeletal muscle TE.
Satellite cells are sequestered between the sarcolemma and mature muscle fibers and normally do not proliferate. In response to specific local challenges such as muscle damage, myogenic satellite cells start to proliferate. They migrate through the basal lamina sheets to areas of injury, differentiate into myoblasts and fuse with pre-existing, damaged fibers or with each other and ultimately differentiate to muscle fibers. Remarkably, if satellite cells fail to fuse they dedifferentiate back to quiescent satellite cells [22]. Myogenic satellite cells can be harvested from muscle tissue, which is easy accessible, and propagated *in vitro*.
Unfortunately, the differentiation processes towards myofibers still appear difficult to induce and control *in vitro*. Therefore, frequently cell lines are used such as C2C12, which are satellite cells of C3H mice [23-29]. Also, satellite cells harvested from the soleus muscle of rats are used for TE of muscle tissue [28,30-36,36,37]. Other muscles harvested for TE research include the latissimus dorsi and rectus femoris of rats [38], flexor digitorum brevis of rats [39], tibialis anterior of rats [23,28,36,36], the extensor digitorum longus of mice [23], the human masseter [40-44] and brachioradialis [45], and the iliofibularis from female Xenopus laevis frogs [46] (Table 2).

Even *in vitro*, the predetermination of satellite cells is preserved. In other words, the source of muscle fiber type predisposes satellite cells differentiation: Huang et al. showed that characteristics of muscle fibers tissue engineered of soleus muscle satellite cells are different from those obtained of tibialis anterior muscle satellite cells. For example, the contraction/relaxation time is slower in muscle constructs derived of satellite cells of soleus muscle compared to muscle constructs derived of satellite

Table 2. Sources of progenitor cells.

Reference	Species	Type	Progenitor cells
Caplan A.I. Dezawa M.	Human Rat	Bone marrow	Mesenchymal stem cell
Lewis MP. Shah R. Sinanan AC. Stern-Straeter J.	Human	Masseter muscle	Satellite cell
Alessandri G.	Human	Brachioradialis muscle	Satellite cell
Lewis MP. Borschel GH. Dennis RG. Huang NF. Levenberg S. Riboldi SA. Boontheekul T.	C3H mice	C2C12	Cell line
Borschel GH. Larkin LM. Dennis RG. Huang NF. Das M. Beier JP. Bach AD. Stern-Staeter J.	Rat	Soleus muscle	Satellite cell
Boontheekul T. Huang YC. Dennis RG. Boontheekul T.	Rat	Tibialis anterior muscle	Satellite cell
Kamelger FS.	Rat	Latissimus dorsi muscle	Satellite cell
Kamelger FS.	Rat	Rectus femoralis muscle	Satellite cell
De Coppi P.	Rat	Flexor digitorum brevis muscle	Satellite cell
Dennis RG.	Mice	Extensor digitorum longus muscle	Satellite cell
Jaspers RT.	Frog	Iliofibularis muscle	Satellite cell

cells of tibialis anterior muscle [47]. This suggests a strong imprinting of myogenic satellite cells.
McLoon et al. suggested that facial muscles contain satellite cells with unique abilities, amongst which a higher quantity and resistance to apoptosis, which renders them more favorable for TE than other skeletal muscle derived satellite cells [48].
A difficulty lies in culturing differentiated skeletal muscle. Sinanan et al. investigated an approach to purify the population of myogenic satellite cells through $CD56^{pos}$, which is a cell adhesion molecule (NCAM) expressed in proliferating myoblasts [49]. Though the resulting cell population has a higher percentage of desmin [41], which is an intermediate filament protein, force production of this population was significantly lower in comparison to the mixed and $CD56^{neg}$ cell populations [43].
Previously mentioned MSCs harbor a side population of $CD31^{neg}$ $CD45^{neg}$ mesenchymal stem cells that reside in bone marrow [50,51], but also skeletal muscle itself [52-55]. A co-culture of myogenic satellite cells and this side population of $CD31^{neg}$ $CD45^{neg}$ mesenchymal stem cells is another approach in stimulating proliferation and differentiation of myogenic satellite cells.
However, hurdles still remain concerning differentiation towards functional skeletal muscle before TE is clinically applicable in patients with facial paralysis.

The proneness of satellite cells for myogenic differentiation, the strong imprinting and the unique characteristics of facial muscles suggest that it is important for the functional outcome in TE of facial muscles to use appropriate progenitor cells, such as satellite cells derived of facial muscle.

SCAFFOLDS

In vivo, the extracellular matrix (ECM) of muscles provides muscle fibers with the architecture to support development and function. Thus, TE of functional skeletal muscle requires a scaffold to mimic the ECM, to support proliferation and differentiation of progenitor cells [38].
Different types of scaffolds have been developed with regard to their physiochemical features and compositions, as well as their biological characteristics (Fig. 1).
Although several studies have addressed the development of an optimal non-biodegradable scaffold, like phosphate based glass [44], biodegradable scaffolds are preferred, because upon degradation, remodeling to the natural muscular ECM can occur.

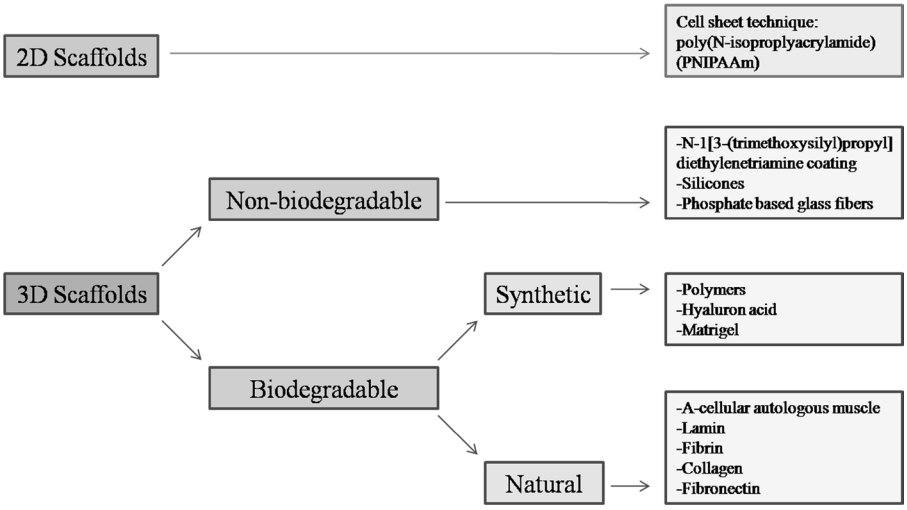


Figure 1. Different designs for scaffolds currently used in muscle TE.

Both synthetic and natural scaffolds have been developed. Synthetic biodegradable three dimensional (3D) scaffolds that hold promise for muscle TE are polymers made of polylactic-co-glycolic acid (PGA) fiber mesh sheets [56]. These have been found to provide both appropriate rigidity and connection. Aligned nanoscale and microscale topographic features of a polymer scaffold both cause alignment of myoblasts and cytoskeletal proteins, and promote myotube assembly along the nanofibers and microgrooves to mimic the myotube organization in muscle fibers. This alignment is an important requirement of functional skeletal muscle. Furthermore, they enhance myotube striation, restrict cell spreading and finally suppress myoblast proliferation during differentiation and cell fusion. Nanoscale features are more efficient in promoting the assembly of longer myotubes than microscale features [27]. Parallel alignment can also be induced by applying mechanical or magnetic strain: muscle fibers reorganize along the line of force [29,57] and through static magnetic fields [58].

Also in a natural biodegradable 3D scaffold, like collagen, aligned topographic features cause alignment of myoblasts and cytoskeletal proteins [59,60]. A natural biodegradable 3D scaffold can also be made of acellular muscle ECM [24]. A drawback of this type of scaffold is its extreme fragility and associated difficulties of handling. Another natural biodegradable 3D scaffold can be created by using fibrin. Satellite cells are mixed with a growth medium, fibrin and thrombin to create a fibrin gel [29,31,32,37,61]. Muscle progenitor cells produce their own ECM proteins and over 3 to 4 weeks degrade and replace the original fibrin matrix [36], which is important

because myoblasts proliferate more extensively in fast degrading gels [28]. This process is much similar to wound healing *i.e.* repair, in which fibrin forms a temporary scaffold to serve tissue regeneration. Upon completion of wound healing *i.e.* repair, the fibrin has been replaced by the physiological ECM. Fibrin has the additional advantage that it binds growth factors, such as fibroblast growth factor (FGF-2), vascular endothelial growth factor (VEGF) and indirectly insulin growth factor (IGF-1), which all augment myogenesis. Myoblasts seeded on fibrin gels have been shown to differentiate into contracting muscle fibers and demonstrate a normal length-tension and force-frequency relationship [36].
In vitro seeding of myogenic progenitor cells on a scaffold has been found to enhance differentiation into muscle fibers after implantation *in vivo* and results in ingrowth of a nourishing capillary network. This might be part of the foreign body reaction that occurs after implantation of a skeletal muscle construct *in vivo* [38,62,63].
An alternative technique which does not require a 3D scaffold is cell sheet technology [64-70]. Although originally developed for myocardial TE purposes [70], the technique has found its way to other fields of TE, like liver [69] and cornea [65], and is promising for engineering facial muscles. It exercises the use of temperature responsive 2D scaffolds made out of polymer: poly(N-isoproplyacrylamide). By increasing the temperature, the cell layer becomes afloat, which allows non-invasive harvesting, *i.e.* without the use of enzymes such as trypsin. By means of a stamp, the cultured cells can be processed as intact sheets, along with their deposited ECM. Out of these cell sheets, 3D constructs can be created by layering these cell sheets (Fig. 2). However, the number of cell sheet layers is limited because developing myoblasts are unable to proliferate and differentiate greater than approximately 150 μm from a nutrient source [30].

The mentioned scaffolds all have their own advantages. Parallel alignment can be reached through aligned topographic features, fibrin promotes cell proliferation, the cell sheet technique offers an excellent architecture of monolayers. Combined, they would seem to form the ideal scaffold for skeletal muscle TE (Fig. 3).

INNERVATION

One of the key features for innervation of a muscle construct is formation of a motor end plate, the neuromuscular junction between the motorneuron and every individual

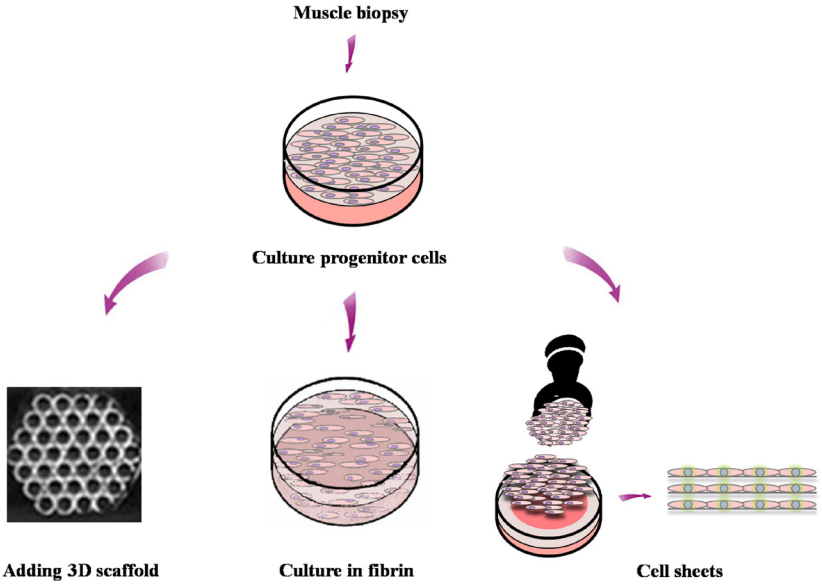


Figure 2. Concept of *in vitro* TE of skeletal muscle with three different types of scaffolds.

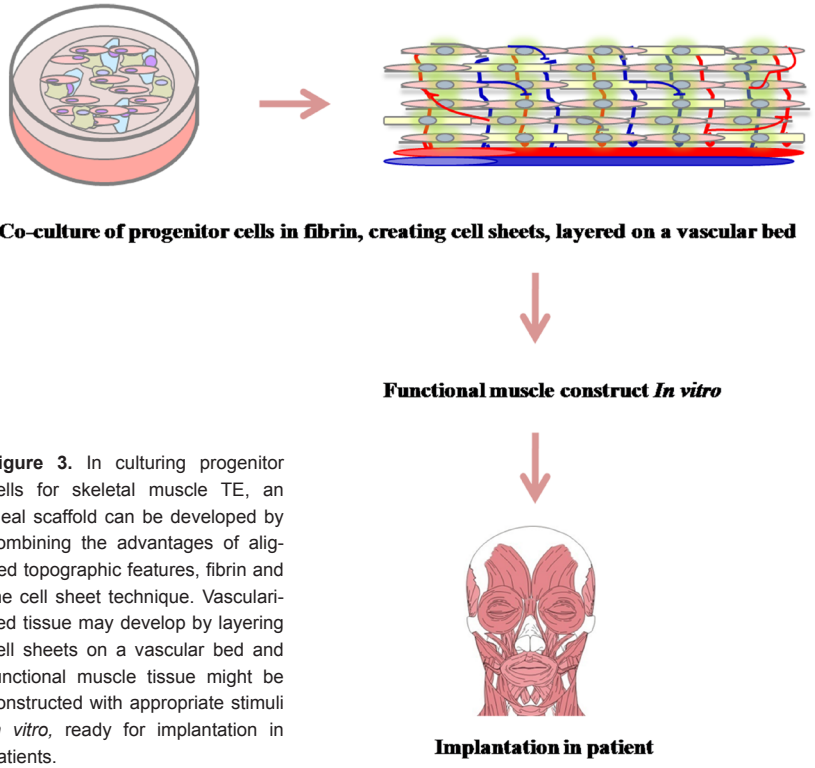


Figure 3. In culturing progenitor cells for skeletal muscle TE, an ideal scaffold can be developed by combining the advantages of aligned topographic features, fibrin and the cell sheet technique. Vascularized tissue may develop by layering cell sheets on a vascular bed and functional muscle tissue might be constructed with appropriate stimuli *in vitro,* ready for implantation in patients.

muscle fiber of the construct.
It is important to understand the influence on, and the effect of chemotropic, electrical and mechanical stimulation on cultured myoblasts with respect to their proliferation and differentiation.

During *in vivo* myogenesis acetylcholine receptors (AChR) are expressed randomly along the surface of myoblasts. Stimulation induces the accumulation of AChR in the plasma membrane of developing muscle fibers [35]. Whenever a motor nerve comes in the vicinity of such an AChR-spot, a motor end plate may develop. Continuous communication between the muscle fibers and the nerve is necessary in maintaining the neuromuscular junction, because these junctions disappear upon loss of communication [71].
In co-cultures of myotubes and neural cells neuromuscular-like junctions appear to form spontaneously. On muscle cells AChR are accumulated and are surrounded by neural extensions that harbor neurofilaments. These nerve-muscle constructs show spontaneous contractions and can also be stimulated [72]. Larkin et al. showed *in vitro* that stimulated nerve-muscle constructs had better contractility characteristics than muscle-only constructs: muscle-only constructs, produced a twitch force of 40 μN, and a tetanic force of 95 μN, while stimulated nerve-muscle constructs produced a twitch force of 100 μN, and a tetanic force of 200 μN [35]. Dhawan et al. showed that *in vivo* neurotization of skeletal muscle constructs led to better contractibility *ex vivo* compared to the non-neurotizated skeletal muscle constructs [73]. This indicated that skeletal muscle constructs display a greater maturity, and thereby a higher force production in the presence of neural cells and neuromuscular junctions.

The most important contraction proteins of muscle fibers are desmin and myosin heavy chain (MHC). Besides, myogenic regulator factors (MRFs), such as MyoD and myogenin, regulate expression of AChR as part of the motor endplate development. It has been found that the expression of desmin, MHC and MRFs can be strongly influenced by chronic electrical stimulation, which imitates *in vivo* neuronal activity during myogenesis *in vitro* [74,75]. Huang et al. used this concept on muscle fiber constructs from soleus and tibialis anterior muscle satellite cells and found an increase in force production of 61–80 % in soleus muscle constructs, without an increase in total MHC [47]. They hypothesized that the increase in force production is caused by reorganization of sarcomeres within muscle fibers promoted by chronic low-frequency electrical stimulation, and/or a change in matrix produced by muscle cells, which allowed for

better transmission of force to the anchor materials, specifically designed to serve as artificial tendons. Unfortunately, this protocol was not found to be universally effective, since it did not show this effect in the tibialis anterior muscle constructs.
This once again emphasizes that characteristics of satellite cells used to generate muscle constructs determine, amongst others, the contractile force of a muscle construct.

Although proof-of-principle experiments showed that electrical stimulation was essential for the formation and maintenance of functional signaling between motor nerve and muscle fiber, other experiments showed that, depending on the nature of this electrical stimulation, also opposite effects can be achieved [71,75].
When contractions exceeded 800 a day, loss of muscle mass and force production was found within 24 hours. *In vivo* this is within the normal range for fast twitch muscle, but apparently in this culture this is inappropriate [47].
It is a fundamental feature of skeletal muscle *in vivo* as well as *in vitro* that optimal force production is a function of stimulation voltage and frequency. Therefore, electrical stimulation needs to be further optimized in terms of frequency, pulse width, and train duration to minimize tissue damage and maximize beneficial phenotypic alterations.

As said before, the formation of the neuromuscular junction is also influenced by chemotropic stimulation. Myoblasts appear to undergo dramatic phenotypic changes upon prolonged culturing and passaging. This was found to be most prominent for the expression of MRFs such as MyoD and myogenin, and thus for AChR expression. Myoblasts that underwent low passage have a higher expression level of MyoD, myogenin and AChR compared to myoblasts that underwent high passage. It also has a diminishing effect on the force production [32]. This indicates that frequent passaging of cells delays synaptic signaling between the motor nerve ending and the muscle fibers.
In conclusion, for developing of a fully functional neuromuscular junction with neural cells, it seems important to apply the optimal electrical, chemotropic and mechanical stimulation and discover other factors that influence its formation.

VASCULARISATION

In contrast to the limited research into innervating tissue engineered skeletal muscle

constructs, there has been extensive research into vascularisation of these constructs. This is very important since a major limiting factor in creating skeletal muscle via TE is that myoblasts are unable to proliferate and differentiate greater than 150 μm from a nutrient source and oxygen supply [30]. Obviously, most muscles are thicker than 300 μm. Thus, every muscle construct must be connected to a vascular system for efficient transport of oxygen, carbon dioxide and nutrients and waste products.

Different options have been studied. First, implantation *in vivo* of muscle constructs gives the foreign body reaction that results in ingrowth of a supportive capillary network that subsequently promotes and maintains viability [61,62,76,77]. Still, to engineer thicker, functional tissue, an integrated vascular system is required.
The second option includes fibrin gel fixed around a preformed ectopic arteriovenous loop in rats [37]. If primary expanded myoblasts are injected into this prevascularised fibrin gel, vascularised muscle tissue is expected to develop. However, Bach et al. showed that, although cells survived and kept their myogenic phenotype, muscle fiber formation was not reached yet. Differentiation of myoblasts failed in these experiments [37], opposite to the experiments of Huang et al., which showed that fibrin gel as a scaffold promotes proliferation and differentiation of myoblasts [36]. It might be that potent stimuli, like chemotropic, electrical and mechanical stimulation, which are necessary for differentiation of myoblasts into muscle fibers, were lacking in the experiments of Bach et al.
Third, Borschel et al. implanted engineered skeletal muscle constructs around femoral vessels in rats. This led to the formation of vascularised skeletal muscle tissue which, when electrically stimulated, produced a longitudinal specific force of 35.4 ± 62.2 N/m^2 [34].
Fourth, Levenberg et al. developed so called prevascularised skeletal muscle constructs from cultures of myoblasts, endothelial cells and embryonic fibroblasts, in which enhancement of the vascular network was perceived. This appears of major interest because these *in vitro* prevascularised muscle constructs showed less apoptosis of cells after implantation *in vivo*, than muscle constructs that were not prevascularised. Reduction of apoptosis is correlated to vessels in muscle constructs that form anastomoses with the host vasculature [25]. Further research is needed to establish whether this model is applicable in humans, the use of a xenogenic culture system (human and animal) in combination with embryonic cells is more than questionable, since embryonic tissue might give rise to malignant proliferation and implantation of animal tissue gives rise to an immunological response.

The cell sheet method mentioned earlier also holds promise to the concerted vascularisation of muscle constructs. By implanting the maximum of cell sheets over a vascular system, neovascularisation occurs *in vivo* [78]. If sufficient neovascularisation occurs, a new layer of cell sheets can be applied on top of the previous, where after neovascularisation of the new layer of cell sheets takes place. Through this kind of polysurgery, which applies the use of repeated transplantations after neovascularisation, thick muscle tissue with connectable vessels may be produced [78-80,80,81]. This technique has not yet been used for engineering skeletal muscle, and current method is not practical in a human situation. Combining the cell sheet technique with a fibrin coating and aligned topographical features, with a co-culture of myogenic satellite cells and endothelial progenitor cells, this technique can be made applicable in a human situation.

CONCLUDING REMARKS AND PERSPECTIVE

Human myogenic satellite cells are at present the first choice for skeletal muscle TE purposes. Characteristics of satellite cells used for TE have a direct effect on characteristics and contractility of resulting muscle tissue. Further research is required to determine whether satellite cells from facial muscles are applicable to tissue engineer new facial tissue and whether a combination with other mesenchymal stem cells is preferred in developing specific characteristics necessary for clinical application in patients with facial paralysis.

A scaffold should promote proliferation of satellite cells, differentiation and parallel alignment. Aligned topographic features, fibrin and the cell sheet technique all have their own advantages, which combined would seem to form an ideal scaffold for engineering functional skeletal muscle.

It seems possible to create a functional neuromuscular junction through co-culture of myotubes and neural tissue, or by means of electrical stimulation. However, innervating the muscle construct remains a major hurdle for clinical applicability of skeletal muscle created via TE.

Vascularisation of the muscle construct is essential to accomplish volume necessary for the construct to be functional in clinical application. This could be reached through *in vivo* implantation of a preformed muscle construct around a vascular pedicle, through implantation of myoblasts in a prevascularised fibrin gel, or through the cell sheet method. These options are currently not suitable for human application and

an *in vitro* model seems to have the most potential. We anticipate that by combining different scaffold techniques and co-culturing different cell types, further process can be made in engineering functional skeletal muscle.
Together these new technologies provide a novel insight in an alternative and improved method for reconstructive surgery in patients with facial paralysis.

CHAPTER

3

A GLOBAL DOWNREGULATION OF MICRORNAS OCCURS IN HUMAN QUIESCENT SATELLITE CELLS DURING MYOGENESIS; IN PARTICULAR OF MICRORNA CLUSTER 106B-25, MICRORNA-29C AND MICRORNA-320C

Merel Koning, Paul M.N. Werker, Marja J.A. van Luyn, Guido Krenning, Martin C. Harmsen

ABSTRACT

During myogenesis, human satellite cells differentiate and form multinucleated myotubes, while a fraction of the human satellite cells enter quiescence. These quiescent satellite cells are able to activate, proliferate and contribute to muscle regeneration. Post-transcriptional regulation of myogenesis occurs through specific myogenic microRNAs, also known as myomiRs. Although many microRNAs are involved in myotube formation, little is known on the involvement of microRNAs in satellite cells entering quiescence. This current study aims to investigate microRNA involvement during differentiation of human satellite cells, specifically proliferating satellite cells entering quiescence.

For this, clonally expanded human satellite cells were differentiated for 5 days, after which myotubes and quiescent satellite cells were separated through FACS sorting. Next, a microRNA microarray comparison of proliferating satellite cells, myotubes and quiescent satellite cells was performed and verified through qRT-PCR.

We show that during human satellite cell differentiation, microRNAs are globally downregulated in quiescent satellite cells compared to proliferating satellite cells, in particular microRNA-106b, microRNA-25, microRNA-29c and microRNA-320c. Furthermore, we show that during myogenesis microRNA-1, microRNA-133, microRNA-206 and microRNA-486 are involved in myotube formation rather than satellite cells entering quiescence. Finally, we show an overall decrease in total mRNA in quiescent satellite cells, and an indication that RNaseL regulation plays a role in promoting and maintaining quiescence. Given the importance of quiescent satellite cells in skeletal muscle development and regenerative medicine, it is imperative to distinguish between myotubes and quiescent satellite cells when investigating skeletal muscle development. Especially in microRNA studies, since we show that microRNAs are globally downregulated in quiescent human satellite cells.

INTRODUCTION

Muscle tissue has its own endogenous repair and maintenance system which is based on myogenic progenitor cells, *i.e.* satellite cells. *In vivo*, satellite cells are activated upon tissue damage, they proliferate and differentiate, fuse with existing myofibers and thereby contribute to the regeneration of damaged muscle [82-86]. During differentiation *in vitro*, part of the human satellite cells form multinucleated myotubes, and the other part enters quiescence [87,88]. While myotubes are terminally differentiated, these quiescent satellite cells, also called reserve cells [89], are still able to activate, proliferate and differentiate to form myotubes and contribute to muscle regeneration and repair. The ability to adapt to two distinct cell fates, *i.e.* towards myotube formation or quiescence in the same microenvironment is unique for satellite cells [90,91]. The myogenic differentiation process is transcriptionally regulated through factors such as Pax7 and the myogenic regulator factors MyoD and Myogenin. While post-transcriptional regulation further occurs through specific myogenic microRNAs, also known as myomiRs [92,93].

MicroRNAs are small, non-coding RNAs, 20-22 nucleotides in length, involved in post-transcriptional gene regulation through inhibition of protein translation or enhancing messenger RNA degradation. MyomiRs have an important role in skeletal muscle development and disease [94-99]. A major effect of microRNAs in myogenesis is that they modulate proliferation as well as differentiation of satellite cells [9]. MicroRNA-133 promotes satellite cell proliferation through repressing the Serum Response Factor [100], but is not essential in skeletal muscle development [101]. MicroRNA-1, microRNA-206 and microRNA-486 are strongly upregulated during satellite cell differentiation and subsequent muscle development. They improve muscle differentiation by inhibiting *PAX7* translation such that *MYOD1* is no longer inhibited and myotube formation progresses [102-105]. Finally, other microRNAs that improve myotube formation are microRNA-181 that targets Hox-A11[106], microRNA-24 [107,108] and microRNA-27 that facilitates the start of myotube formation by targeting *PAX3* [109]. Also microRNA-29 improves myotube formation by targeting *HDAC4* [110], thereby preventing muscle degeneration [111].

Although many microRNAs are involved in myotube formation, little is known on the involvement of microRNAs in satellite cells entering quiescence, which is of importance in skeletal muscle regeneration. This current study aims to investigate microRNA involvement during differentiation of human satellite cells through a microRNA microarray of proliferating satellite cells, myotubes and quiescent satellite

cells to elevate the knowledge of microRNAs during differentiation of human satellite cells.

MATERIALS AND METHODS

Satellite cell isolation and culture

A muscle biopsy was obtained from a healthy female donor, undergoing blepharoplasty. The age of the donor was 60 years. The study protocol was approved by the institutional medical ethics committee, and the donor gave her informed consent. Satellite cells were isolated with 0.04 mg/ ml (0.16 Collagenase Wünsch units/ml) Liberase Blendzyme 3 (Roche Applied Science, the Netherlands) as described previously [112]. Proliferation medium consisted of Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen/Gibco, CA, USA), 20 % Fetal Bovine Serum (FBS; Invitrogen/Gibco) and 1 % penicillin/streptomycin 50 µg/ml (Sigma-Aldrich, St. Louis, USA). Differentiation medium (DM), contained DMEM, 2 % FBS, 1 % penicillin/streptomycin, 1 % Insulin-Transferrin-Selenium-A (100x) (Invitrogen) and 0.4 µg/ml dexamethason (Sigma-Aldrich). Medium was refreshed three times per week. Cells were plated at 5.0 x 10^3 cells/cm^2 in culture flasks precoated with 1 % gelatine/PBS for 30min. When cells reached 70 % confluence, they were enzymatically harvested using accutase (Invitrogen) and passaged. Passage number (P_x) was defined as the xth sequential harvest of a subconfluent cell population. At passage 8, cells were cloned by sorting single cells in 96 wells plates using a MoFlow FACS. The fraction of human satellite cells that was able to clonally expand was 26.3 ± 4.0 %. A clone that uniformly expressed the satellite cell marker Pax7 and the myogenic regulator factors MyoD and Myogenin was selected for further experiments.

After 5 days differentiation, we sorted mononuclear cells and myotubes by first harvesting the whole cellfraction using accutase (Invitrogen), then sieving the cellfraction through a 70 µm sieve. Next, we stained the flowthrough with 5 µg/ml Hoechst (Invitrogen) for 30 min at room temperature and finally we performed sorting by FACS to obtain the mononucleated fraction. Proliferating satellite cells were handled similarly, and myotubes were retrieved of the sieve filter.

Immunofluorescent staining

Cells were cultured on Thermanox® coverslips, Lab-Tek chamber slides or 96 wells plates (all NUNC Brand Products, Roskilde, Denmark) coated with 1 % gelatine. At

100 % confluence, cells were fixed or cultured for an additional five days in DM and subsequently fixed in 2 % paraformaldehyde (PFA) at room temperature for 10 min. A permeabilization step was performed with 0.5 % Triton X-100 (Sigma-Aldrich) in PBS at room temperature for 10 min. Non-specific binding-sites were blocked with 10 % Goat serum in PBS for 30 min. Cells were incubated with the primary antibody in PBS and 2 % serum at room temperature for 60 min or at 4 °C overnight. The primary antibody consisted of either a proliferation marker, rabbit-anti-human Ki67 (1:100) (Sanbio, Uden, the Netherlands), a satellite cell marker, mouse-anti-human Pax7 (1:10) (Developmental Studies Hybridoma Bank (DSHB), Iowa, USA), a myogenic marker, rabbit-anti-human desmin (1:100) (Novus Biological, Littleton, USA), a myogenic transcription factor, mouse-anti-human MyoD (1:100) (Dako, Glostrup, Denmark), a myogenic transcription factor, mouse-anti-human myogenin (1:100) (DSHB), a sarcomere component, mouse-anti-human myosin (MF20; 1:500) (DSHB) and mouse-anti-human RNaseL (1:100) (Abcam, Cambridge, UK). After three washes with 0.05 % Tween in PBS the cells were incubated with a secondary antibody-cocktail at room temperature for 30min. The secondary antibody-cocktail constituted of FITC-conjungated goat-anti-rabbit IgG (1:100) (Southern Biotech, AL, USA), Alexa Fluor®488 goat-anti-mouse IgM and Alexa Fluor® 555 goat-anti-mouse IgG1 or IgG2b (all Invitrogen) (1:300 in PBS/DAPI containing 10 % normal human serum). Samples were mounted in Citifluor AP1 (Agar Scientific, Essex, UK). Examination was performed by immunofluorescent microscopy using a Leica DMRXA microscope and Leica Software (Leica Microsystems, Wetzlar, Germany), and further quantification was performed by TissueFAXS using a Zeiss AxioObserver.Z1 microscope and TissueQuest Cell Analysis Software (TissueGnostics, Vienna, Austria).

Gene transcript analysis

Total RNA was isolated from approximately 200,000 cells using the Rneasy Kit (Qiagen Inc., CA, USA), in accordance to the manufacturer's protocol. Briefly, a cell lysate was made and diluted with an equal volume of ethanol (70 %). RNA was collected on an RNA binding filter by centrifugation. DNA was removed by incubation with a DNase I solution at 37 °C for 15 min. The RNA-binding filter was washed twice and subsequently the RNA was eluted with 14 µl Elution Buffer. The RNA concentration and purity were determined by spectrophotometry (NanoDrop Technologies, Wilmington, NC). For qRT-PCR analysis, total RNA was reverse transcribed using the First Strand cDNA synthesis kit (Fermentas UAB, Lithuania). In summary, 1µg of total RNA was diluted in a final reaction volume of 20µl containing random

hexamer primer (0.5 µg), RiboLock™ Ribonuclease Inhibitor (20 U), 1 mM dNTP mix, and incubated at 37 °C for 1 h. The reverse transcription reaction was terminated by heating the mixture to 70 °C for 10 min, after which the samples were placed on ice. Quantitative RT-PCR analysis was performed in a final reaction volume of 10 µl, consisting of SYBR Green Supermix (Bio-Rad, Hercules, USA), 0.5 mM primer-mix (Table 1) and 5 ng cDNA. Reactions were performed at 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s, for 40 cycles. Analysis of the data was performed using Science Detection Software 2.2.2. To determine differences in expression, Ct-values were normalized against GAPDH-expression using the ΔCt-method ($\Delta Ct_{(gene)} = Ct_{(gene)} - Ct_{(GAPDH)}$). Relative expression levels were calculated as $2^{-(\Delta Ct)}$. All cDNA samples were amplified in triplicate.

Table 1. Primer sequence quantitative reverse transcription polymerase chain reaction.

Primer	Forward	Reverse
PAX7	ATCCGGCCCTGTGTCATCTC	CACGCGGCTAATCGAACTCA
MYOD1	AGCACTACAGCGGCGACTCC	CACGATGCTGGACAGGCAGT
MYL1	AAGCCCGCAATGCAGAAGAG	TTGCTTGCAGTTTGTCCACCA
MYL3	GAACACCAAGCGTGTCATCCA	TCAGCAGATGCCAGTTTTCCA
RNASEL	CTGGCAGATTTTGATAAGAGCA	ATAGAGGACCAGCCGTCCA
GAPDH	CTGCCGTCTAGAAAAACCTG	GTCCAGGGGTCTTACTCCTT

MicroRNA analysis

Total RNA was isolated from approximately 200,000 cells using the mirVana kit (Ambion), in accordance to the manufacturer's protocol. Briefly, a cell lysate was made and diluted with 1.25 volumes of ethanol (100 %). RNA was collected on a RNA binding filter by centrifugation. The RNA concentration and purity were determined by spectrophotometry (NanoDrop Technologies, Wilmington, NC).

Microarray analysis of proliferating satellite cells, myotubes and quiescent satellite cells (N = 3) was performed according to the manufacturer's protocol (Agilent, Santa Clara, SA). Briefly, first strand cDNA was synthesized from 100 ng RNA, followed by cRNA amplification and labeling with Cy3. Samples were hybridized in a dye swap design at 65 °C overnight on Agilent microRNA Microarray 16.0. The following day, slides were washed and signals were scanned with GenePix 4000B (Agilent). Signal intensities from scanned images were processed and converted into normalized data using Agilent Feature Extraction software version 9.1. Samples were analyzed and

a quality control report was generated using GeneSpring GX version 9.0 (Agilent). cDNA synthesis was performed using the microRNA Reverse Transcription Kit (Applied Biosystems). In summary, 25 ng of total RNA was diluted in a final reaction volume of 5 µl with 1.25 µl 6 µM microRNA specific RT-primer (Table 2) and 1.25 µl RT-master mix, containing 1mM dNTP mix, multiscribe RT enzyme, RT Buffer, RNase Inhibitor and water. This was incubated at 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min, and subsequently mixed with 0,5 µl 10 µM microRNA specific qRT-primer and 0,5 µl 10 µM reverse primer (Table 2). Quantitative RT-PCR analysis was performed with 25 ng cDNA-primer mix and 4 µl SYBR Green Supermix (Bio-Rad, Hercules, USA). Reactions were performed at 95 °C for 15 s, 60 °C for 60 s, for 45 cycles. Analysis of the data was performed using Science Detection Software 2.2.2. To determine differences in expression, Ct-values were normalized against RNU6B-expression using the ΔCt-method ($\Delta Ct_{(microRNA)} = Ct_{(microRNA)} - Ct_{(RNU6B)}$). Relative expression levels were calculated as $2^{-(\Delta Ct)}$. All cDNA samples were amplified in triplicate.

Table 2. Mature microRNA primer sequence for qRT-PCR.

microRNA	Stemloop Primer	Forward Primer
mir-106b	GTCGTATCCAGTGCAGGGTCCGAGG-TATTCGCACTGGATACGACATCTGCACT	TGCGGTAAAGTGCTGAC
mir-25	GTCGTATCCAGTGCAGGGTCCGAGG-TATTCGCACTGGATACGACTCAGACCGAG	TGCGGCATTGCACTTGT
mir-29c	GTCGTATCCAGTGCAGGGTCCGAGG-TATTCGCACTGGATACGACTAACCGATTT	TGCGGTAGCACCATTTG
mir-320c	GTCGTATCCAGTGCAGGGTCCGAGG-TATTCGCACTGGATACGACACCCTCTC	TGCGGAAAAGCTGGGTT
RNU6B	GTCGTATCCAGTGCAGGGTCCGAGG-TATTCGCACTGGATACGACAAAAATATGG	TGCGGCTGCGCAAG-GATGA
Reverse Oligo	GTGCAGGGTCCGAGGT	

Statistics

All data are represented as mean ± SEM and statistical comparison between two groups was performed using the Student's t test. When comparing more than two groups one way ANOVA was used followed by the post hoc Bonferroni's multiple comparison test in Graph-Pad Prism Version 5 (GraphPad Software, Inc., La Jolla, CA, USA). Results were considered significant for $p < 0.05$.

RESULTS

Satellite cells differentiate into myotubes or enter quiescence.

Human satellite cells were isolated and cultured from enzymatically dissociated muscle tissue. Initial passages comprised of heterogeneous cell populations. At passage 8, satellite cells were cloned by single cell sorting. Differential Interference Contrast imaging showed proliferating satellite cells at passage > 8, and after 5 days of differentiation when part of the satellite cells fused and formed myotubes while another part remained mononuclear (Fig. 1A-B). Clonal satellite cells uniformly expressed Pax7 and Desmin during proliferation (Fig. 1C). Five days after differentiation, Pax7 positive nuclei were limited to, and increased in the remaining mononuclear satellite cells (Fig. 1D). Desmin expression on the other hand, was increased in myotubes.

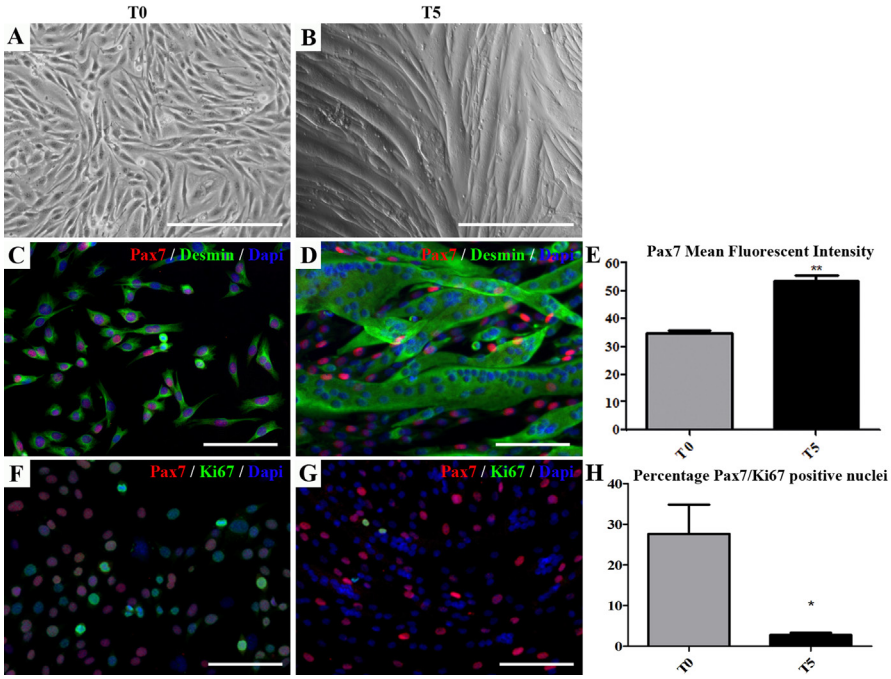


Figure 1. Human satellite cells differentiate into myotubes or go into quiescence. (A-B) Differential Interference Contrast image of (A) cloned proliferating satellite cells (T0) and (B) at day 5 after differentiation (T5). (C-D) Immunofluorescent staining for Pax7 (red) and Desmin (green) of (C) proliferating satellite cells and (D) at day 5 after differentiation. All satellite cells at T0 express both Pax7 and Desmin. After differentiation only the mononuclear satellite cells are highly positive for Pax7 while the myotubes are highly positive for Desmin. (E) Analyses by TissueFAXS shows that the mean fluorecent intensity of Pax7 per nuclei increases after differentiation. (F-G) Immunofluorescent staining for Pax7 and Ki67 (green) of (F) proliferating satellite cells and (G) 5 days after differentiation. (H) Analyses by TissueFAXS shows that the percentage Pax7/Ki67 positive nuclei decreased after differentiation. Nuclei are counterstained with DAPI (blue). Scalebars are 100 µm (N = 3; data are represented as means ± SEM; * p = 0.04).

During proliferation the percentage Ki67/Pax7 double positive nuclei was 27.7 ± 7.2 %. Five days after differentiation however, the percentage Ki67/Pax7 double positive nuclei decreased to 2.8 ± 0.8 % (Fig. 1E-G; * p = 0.04).

Myotubes and quiescent satellite cells have distinct regulatory factors

While the expression of Pax7 was limited to nuclei of mononuclear quiescent satellite cells after differentiation (Fig. 1D), the expression of the transcription factors MyoD and Myogenin was increased in nuclei in myotubes (Fig. 2A-B). Also the adult muscle marker Myosin was increased in myotubes (Fig. 2C). After differentiation, we

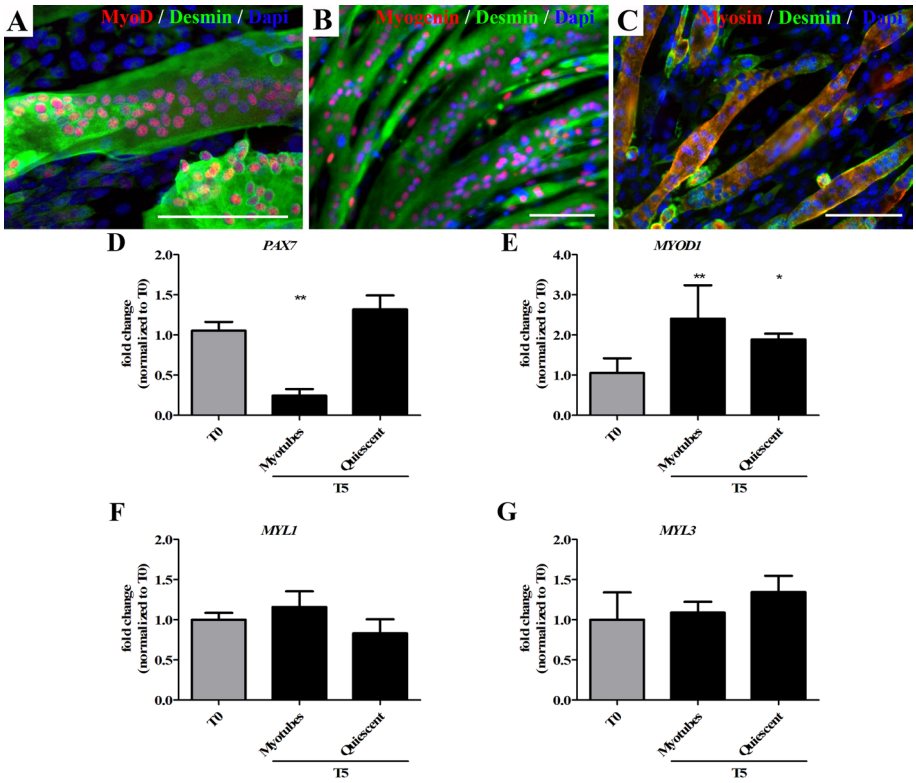


Figure 2. Myotubes and quiescent satellite cells have distinct regulatory factors. (A) Immunofluorescent staining of differentiated satellite cells for MyoD (red) and Desmin (green) shows that only the nuclei of myotubes expressed MyoD. (B) Immunofluorescent staining of differentiated satellite cells for Myogenin (red) and Desmin (green) shows that only the nuclei of myotubes expressed Myogenin. (C) Immunofluorescent staining of differentiated satellite cells for Myosin (red) and Desmin (green) shows that myotubes express both Myosin and Desmin. Nuclei are counterstained with DAPI (blue). Scalebars are 100 µm. (D) Quantitative gene expression analysis of proliferating satellite cells, myotubes and quiescent satellite cells shows a decreased *PAX7* expression in myotubes compared to proliferating satellite cells, (E) an increased *MYOD1* expression in both myotubes and quiescent satellite cells compared to proliferating satellite cells, (F) and no significant change in *MYL1* and (G) *MYL3* expression. (N = 6; data are represented as means ± SEM; * p = 0.02; ** p < 0.01).

separated myotubes from quiescent satellite cells, sorted proliferating satellite cells and quiescent cells, and analysed the three cell fractions by qRT-PCR. In myotubes *PAX7* expression had decreased 0.24 ± 0.08 fold compared to proliferating satellite cells, and *MYOD1* expression had increased 2.41 ± 0.23 fold compared to proliferating satellite cells (Fig. 2D-E; ** $p < 0.01$). In quiescent satellite cells however, *PAX7* expression did not change significantly, while *MYOD1* expression also increased 1.89 ± 0.14 fold compared to proliferating satellite cells (* $p = 0.02$). Though Myosin expression increased in myotubes at protein level, gene expression of both *MYL1* and *MYL3* did not change significantly in either myotubes or quiescent satellite cells compared to proliferating satellite cells (Fig. 2C; F-G).

MicroRNA expression in myotubes and quiescent satellite cells

After differentiation, we separated myotubes from quiescent satellite cells, and sorted both proliferating satellite cells and quiescent cells. We compared the microRNA expression from these cell fractions by Agilent microRNA microarray. After differentiation of satellite cells, we identified four microRNAs that were highly upregulated in myotubes compared to proliferating satellite cells (Fig. 3A). In quiescent cells on the other hand, we did not find any significantly upregulated microRNAs compared to proliferating satellite cells (Fig. 3B). Strikingly, in general all microRNAs were decreased in quiescent cells compared to proliferating satellite cells. Among these were microRNA-106b, microRNA-25, microRNA-29c and microRNA-320c that were significantly downregulated (Table 3).

The downregulation of these microRNAs was confirmed by qRT-PCR; microRNA-106b decreased 0.23 ± 0.15 fold, microRNA-25 decreased 0.42 ± 0.13 fold, microRNA-29c decreased 0.29 ± 0.15 fold, microRNA-320c decreased 0.18 ± 0.06 fold (Fig. 4; ** $p < 0.01$; * $p < 0.05$).

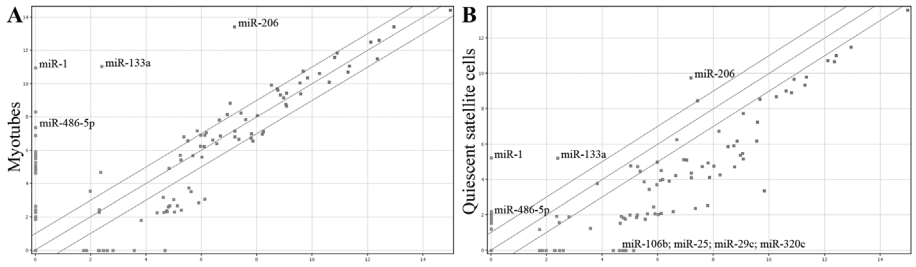


Figure 3. MicroRNA microarray comparing proliferating satellite cells to myotubes and quiescent satellite cells. In myotubes microRNA-1, microRNA-133, microRNA-206 and microRNA-486 are significantly upregulated compared to proliferating satellite cells. Quiescent satellite cells show a global downregulation of microRNAs, significantly microRNA-106b, microRNA-25, microRNA-29c and microRNA-320c (N = 3).

Table 3. Fold change of microRNA expression in myotubes and quiescent satellite cells compared to proliferating satellite cells.

	Myotubes	Mononuclear
mir-1	2002.59 ($p = 7.68*10^{-3}$)	ns
mir-133a	316.85 ($p = 2.38*10^{-4}$)	ns
mir-206	75.80 ($p = 1.79*10^{-3}$)	ns
mir-486-5p	33.91 ($p = 5.73*10^{-3}$)	ns
mir-106b	ns	-27.71 ($p = 1.57*10^{-6}$)
mir-25	ns	-34.82 ($p = 6.80*10^{-5}$)
mir-29c	ns	-24.27 ($p = 1.71*10^{-6}$)
mir-320c	ns	-28.-84 ($p = 2.77*10^{-5}$)

RNaseL involvement in quiescent satellite cells

Besides this overall decrease of microRNA expression in quiescent satellite cells, we also found that total mRNA had decreased. In quiescent satellite cells mRNA decreased 0.29 ± 0.07 fold compared mRNA in to the same number of proliferating satellite cells ($p = 0.02$). Therefore, another post-transcriptional mechanism might be involved in regulating quiescence in satellite cells. RNaseL is an activatable endonuclease that cleaves single-stranded viral, ribosomal, and mRNAs and thus causes an overall reduction of protein synthesis [113]. Overexpression of active RNaseL prevents myotube formation [114,115]. Thus, we argued that RNaseL could also be involved in maintaining or promoting quiescence in satellite cells.

In quiescent satellite cells RNaseL was located in the nuclei (Fig. 5A, arrowheads), while RNaseL was not detectable in the nuclei of myotubes (Fig. 5A, arrow). We also analysed *RNASEL* by qRT-PCR in the separated cell fractions, proliferating satellite cells, myotubes and quiescent satellite cells. The *RNASEL* expression had decreased 0.08 ± 0.03 fold in myotubes compared to proliferating satellite cells (*** $p < 0.001$), while quiescent satellite cells it did not change significantly compared to proliferating satellite cell (Fig. 5B).

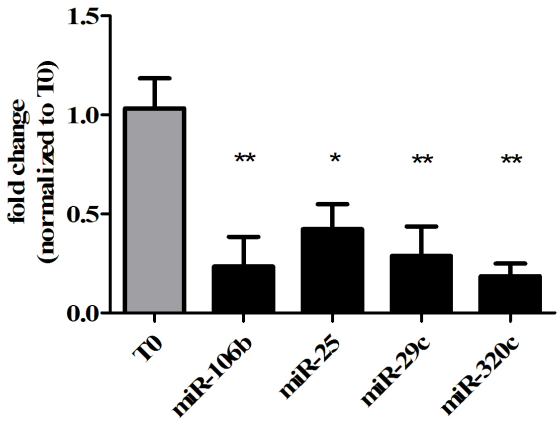


Figure 4. MicroRNA expression in quiescent satellite cells. Quantitative microRNA expression analysis in proliferating satellite cells and quiescent satellite cells shows a decreased expression of microRNA-106b, microRNA-25, microRNA-29c and microRNA-320c in quiescent satellite cells compared to proliferating satellite cells. (N = 6; data are represented as means ± SEM; * p = 0.01; ** p < 0.01).

DISCUSSION

In the current study, we investigated microRNA involvement in the differentiation of human satellite cells. Our main finding is that during differentiation of human satellite cells, microRNAs are globally downregulated in quiescent satellite cells, while in myotubes the expression of myomiRs is increased.

Another major finding is that upon differentiation, Pax7 positive cells stop proliferating and enter quiescence (Fig. 1 C-G). Previously we have shown that Pax7 expression per cell increases [112]. Taken together, our results suggest that two distinct populations of Pax 7-expressing satellite cells exist, that represent different stages: proliferative satellite cells versus non-proliferative satellite cells. These non-proliferative satellite cells arise during myogenesis. They likely have *in vivo* counterparts which are known as reserve cells [89] or quiescent satellite cells [85,90,91,116,117]. *In vivo*, these quiescent satellite cells are important in the maintenance of the regenerative capacity of skeletal muscle. They are capable of proliferation, and either differentiate to repair damaged muscle fibers or enter quiescence to maintain the satellite cell pool. Therefore, investigating the regulation of both these distinct cell fates of satellite cells, *i.e.* myotube formation or entering quiescence, is important for regenerative medicine. However, to our knowledge, microRNA expression in quiescent satellite cells had not been addressed yet. Furthermore, studies on microRNAs in differentiating satellite cells do not distinguish myotubes from quiescent satellite cells. Therefore, the microRNAs which have been previously described to be involved in satellite cell differentiation, *i.e.* myomiRs such as microRNA-1 [105], micro-RNA-133 [100], microRNA-206 [92,102] and microRNA-486 [104], are in fact involved in myotube

formation. We confirmed this, moreover, by showing that these microRNAs are not involved in quiescent satellite cells as such (Fig. 3; Table 3).

Our study shows that both proliferation and microRNA-106b and microRNA-25 are significantly decreased in quiescent human satellite cells. In neural stem/progenitor cells isolated from adult mice, it has been shown that an overexpression of the microRNA-106b ~25 cluster is involved in promoting proliferation, whereas a knockdown of this cluster decreases proliferation [118]. However, in human HEK293 cells, microRNA-25 itself inhibits proliferation [119]. In our study both microRNA-25 and proliferation are decreased. Therefore, the effect of microRNA-25 on the proliferation of human satellite cells remains unclear.

It has previously been shown that in primary mice myoblasts and in the murine myoblast cell line C2C12, microRNA-29c promotes myogenesis [110,111]. It has also been shown that microRNA-29c and microRNA-320 promote apoptosis in myocardial cells [120]. Our study shows that in quiescent human satellite cells, both microRNA-29c and microRNA-320c are decreased; therefore it seems feasible that in quiescent satellite cells both myogenesis and apoptosis are not promoted while quiescence is maintained.

In C2C12 cells it had been shown that RNaseL overexpression inhibits myotube formation [114,121]. RNaseL ia activated when a family of 2',5'-oligoadenylate synthetases bind latent RNase-L resulting in its dimerization and subsequent activation. This activated RNaseL cleaves single-stranded viral, ribosomal, and mRNAs [113].

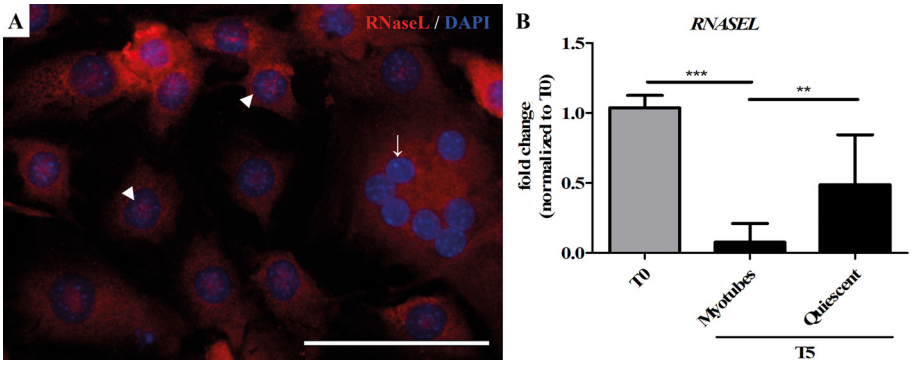


Figure 5. RNaseL expression in quiescent satellite cells. (A) Immunofluorescent staining of differentiated satellite cells for RNaseL (red) shows that only nuclei of quiescent satellite cells express RNaseL(arrowhead), while the nuclei of myotubes do not (arrow). Nuclei are counterstained with DAPI (blue). Scalebar is 100 µm. (B) Quantitative gene expression analysis of RNASEL in proliferating satellite cells, myotubes and quiescent satellite cells shows a decreased expression in myotubes compared to both proliferating and quiescent satellite cells. (N = 6; data are represented as means ± SEM; ** $p < 0.01$; *** $p < 0.001$).

In addition to its nuclease function, a role for RNase-L in translational regulation was recently reported [122]. Furthermore, genetic approaches to manipulate RNaseL expression and activity revealed that it exerts potent antiproliferative, proapoptotic, and senescence inducing activities [123-126]. Since RNaseL is involved in degrading various RNAs, it might also be involved in microRNA degradation. This might explain part of the global downregulation of microRNAs in quiescent satellite cells. On the other hand, it might be that due to the global downregulation of microRNAs that RNaseL is no longer degraded, and therefore quiescence is induced. Anyhow, the nuclear expression of RNaseL in quiescent satellite cells as opposed to myotubes indicates RNaseL involvement in either inhibiting myotube formation or maintaining quiescence.

Although RNaseL might play an active role in the global downregulation of microRNAs in quiescent satellite cells, further research should focus on finding the 'master switch', or other contributing players, that causes this massive downregulation. Little is known about a global microRNA downregulation during differentiation, however, indications that such a 'master switch' might play a role can also be found in oocytes and T-cells. Early during development microRNAs are suppressed in oocytes. This is likely an early event in reprogramming gene expression during the transition of a differentiated oocyte into pluripotent blastomeres of an embryo [127,128]. During antigen induced T-cell differentiation the microRNA profile changes dynamically; also in effector T-cells a global downregulation of microRNAs is observed [129].

CONCLUSION

In the current study, we show that microRNAs are globally downregulated in human satellite cells entering quiescence compared to proliferating human satellite cells. Furthermore, several microRNAs which have previously been described to be involved in satellite cell differentiation are involved in myotube formation rather than satellite cells entering quiescence. Given the importance of quiescent satellite cells in skeletal muscle development and regeneration, it is imperative to distinguish between myotubes and quiescent satellite cells when investigating skeletal muscle development. Especially in microRNA studies, since this study shows that microRNAs are globally downregulated in quiescent human satellite cells; in particular microRNA-106b, microRNA-25, microRNA-29c and microRNA-320c.

CHAPTER

4

MICRORNA-1 AND MICRORNA-206 IMPROVE DIFFERENTIATION POTENTIAL OF HUMAN SATELLITE CELLS: A NOVEL APPROACH FOR TISSUE ENGINEERING OF SKELETAL MUSCLE

Merel Koning, Paul M.N. Werker, Daisy W.J. van der Schaft, Ruud A. Bank, Martin C. Harmsen

TISSUE ENG PART A. 2012 MAY;18(9-10):889-898

ABSTRACT

Innovative strategies based on regenerative medicine, in particular tissue engineering of skeletal muscle, are promising for treatment of patients with skeletal muscle damage. However, the efficiency of satellite cell differentiation *in vitro* is suboptimal. MicroRNAs are involved in the regulation of cell proliferation and differentiation. We hypothesized that transient overexpression of microRNA-1 or microRNA-206 enhances the differentiation potential of human satellite cells by downregulation quiescent satellite cell regulators, thereby increasing myogenic regulator factors.

To investigate this, we isolated and cultured human satellite cells from muscle biopsies. First, through immunofluorescent analysis and qRT-PCR, we showed that in satellite cell cultures low Pax7 expression is related to high MyoD expression upon differentiation, and subsequently more extensive sarcomere formation, *i.e.* muscle differentiation, was detected. Second, using qRT-PCR, we showed that microRNA-1 and microRNA-206 are robustly induced in differentiating satellite cells. Finally, a gain-of-function approach was used to investigate microRNA-1 and microRNA-206 potential in human satellite cells to improve differentiation potential. As a proof of concept this was also investigated in a three dimensional bio-artificial muscle construct. After transfection with microRNA-1, the number of Pax7 expressing cells decreased compared to the microRNA-scrambled control. In differentiated satellite cell cultures transfected with either microRNA-1 or microRNA-206, the number of MyoD expressing cells increased and α-sarcomeric actin and myosin expression increased compared to microRNA-scrambled control cultures. Also in a three dimensional bio-artificial muscle construct an increase in MyoD expression occurred.

INTRODUCTION

Skeletal muscle tissue damaged by prolonged denervation, caused by facial paralysis, often requires extensive surgical reconstruction. Autologous donor muscle can be utilized to repair some muscle damage. However, only one or two muscles can be reanimated, patients are often hindered by autostatic syndrome [5], and genuine regeneration is not achieved [6,7]. Innovative strategies based on regenerative medicine, in particular tissue engineering of skeletal muscle, are promising for treatment of patients with facial paralysis. Engineered muscle tissue may be customized according to the individual patient's desire and offers an opportunity for patients to improve physical and psychological symptoms, without causing significant scarring and donor site morbidity.

Muscle tissue has its own endogenous repair and maintenance system which is based on myogenic progenitor cells, *i.e.* satellite cells. Upon stimuli, such as damage, satellite cells proliferate and differentiate, which contributes to regeneration of damaged muscle [82-85]. The regenerative capacities of human satellite cells derived of skeletal muscle appear to make them a suitable source for tissue engineering [130]. However, myogenesis, *i.e.* efficient differentiation of human satellite cells towards adult skeletal muscle, remains a major hurdle *in vitro*. Moreover, with increasing age, the population of satellite cells per myofiber decreases [131,132]. Furthermore, the myogenic capacity of satellite cells *in vitro* decreases [132-134]. Therefore, tissue engineering of skeletal muscle from autologous satellite cells will be impaired for elderly patients. Due to donor variation, the efficiency of tissue engineering of skeletal muscle will vary between individual patients [135,136]. Therefore, novel approaches to improve myogenesis are mandatory to augment tissue engineering of human skeletal muscle. The process of myogenesis is strongly regulated by epigenetic factors, in particular by microRNAs [94,98,100,137,138]. Therefore, we hypothesized that microRNAs could contribute to improve tissue engineering of human skeletal muscle for future clinical application.

MicroRNAs are small, non-coding RNAs, 20-22 nucleotides in length, involved in post-transcriptional gene regulation through inhibition of protein translation or enhancing messenger RNA degradation. Thereby, they also offer great potential as a tool to modify cell fate and function [9]. MicroRNA-1 and microRNA-206 appear prominent in myogenesis through regulation of the pairedbox genes *PAX3* and *PAX7* [102-104]. These pairedbox genes are quiescent satellite cell regulators which are essential in myogenesis. They function upstream of both myogenic regulator genes *MYOD1*

and *MYF5* to initiate proliferation and muscle differentiation [84,139-141]. If Pax7 is either overexpressed in satellite cells, or if its expression is prolonged, MyoD expression is inhibited and the onset of myogenesis delayed, which prevents muscle differentiation [142]. In satellite cells, microRNA-1 and microRNA-206 downregulate Pax7 such that *MYOD1* is no longer inhibited, and muscle differentiation progresses. Another microRNA involved in myogenesis is microRNA-133 that promotes proliferation through repressing Serum Response Factor, which results in inhibited muscle differentiation [100].

We propose to modify the microRNA profile of satellite cells to facilitate tissue engineering of skeletal muscle, which is a novel concept in regenerative medicine. It offers an opportunity to transfect satellite cells with a pre-microRNA or anti-microRNA in order to modulate myogenesis. By transfecting murine satellite cells with anti-microRNA-133, the contractile force of a bio-artificial muscle increased [143]. However, the potential of human satellite cells transfected with microRNA-1 or microRNA-206 in tissue engineering remains to be investigated.

This current study aims to investigate whether modulation of human satellite cells through microRNA-1 and microRNA-206 could contribute to the differentiation potential of human satellite cells in a three dimensional bio-artificial muscle model, thereby improve tissue engineering of human skeletal muscle.

Our hypothesis is that transient overexpression of microRNA-1 or microRNA-206 enhances the differentiation potential of human satellite cells by downregulation quiescent satellite cell regulators, thereby increasing myogenic regulator factors.

MATERIALS AND METHODS

Satellite cell isolation and culture

Muscle biopsies were obtained from 6 healthy donors, undergoing reconstructive surgery. The age of the donors was 49.5 ± 8 years (30 to 60 years). The study protocol was approved by the institutional medical ethics committee, and patients gave their informed consent. Satellite cells were isolated with 0.04 mg/ ml (0.16 Collagenase Wünsch units/ml) Liberase Blendzyme 3 (Roche Applied Science, the Netherlands) as described previously [112]. Proliferation medium consisted of Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen/Gibco, CA, USA), 20 % Fetal Bovine Serum (FBS; Invitrogen/Gibco) and 1 % penicillin/streptomycin 50 µg/ml (Sigma-Aldrich, St. Louis, USA).

Differentiation medium (DM), contained DMEM, 2 % FBS, 1 % penicillin/streptomycin, 1 % Insulin-Transferrin-Selenium-A (100x) (Invitrogen) and 0.4 µg/ml dexamethason (Sigma-Aldrich). Medium was refreshed three times per week. Cells were plated at 5.0 x 10^3 cells/cm^2 in culture flasks precoated with 1 % gelatine/PBS for 30 min. When cells reached 70 % confluence they were enzymatically harvested using Accutase (Invitrogen) and passaged.
Passage number (P_x) was defined as the xth sequential harvest of a subconfluent cell population. All experiments were performed using P8-15.

Transfection with microRNA-1 and microRNA-206

For gain-of-function studies, pre-microRNA molecules specific for the mature human microRNA-1 sequence, UGGAAUGUAAAGAAGUAUGUAU (pre-miR for hsa-miR-1, PM10617), and microRNA-206 sequence, UGGAAUGUAAGGAAGUGUGUGG (pre-miR for hsa-miR-206, PM10409; both Ambion/Applied Biosystems, Foster City, CA), were transfected with siPORT NeoFX transfection agent (Ambion) into satellite cells in accordance to the manufacturer's protocol. Briefly, the transfection agent was diluted in Opti-MEM I (Gibco) and after 10 min mixed with 50 nM of the microRNA, or with 50 nM scrambled microRNA control (a random, inert nucleic acid sequence). After incubating for 15 min, the mixture was dispensed into gelatin precoated tissue culture flasks. Cells were added to each flask at 1.0 x 10^4 cells/cm^2, and cultured in PM for 24 hours. After 24 hours, PM was refreshed and cells were cultured another 24-48 hours until they reached 100 % confluence.

Bio-artificial muscle construct engineering

Bio-artificial muscle construct were cultured as previously described [144]. Briefly, house-shaped pieces of Velcro were glued to the bottom of a 6 wells plate 12 mm apart, sterilized with ethanol (70 %) and exposure to UV for 15 min. A gel mixture was prepared on ice by mixing 50 % Collagen type I (3,44 mg/ml, BD Biosciences), 39 % PM, 3 % 0.5 M NaOH (Sigma-Aldrich), 8 % growth factor reduced Matrigel® (BD Biosciences). Satellite cells transfected with microRNA-1, microRNA-206 or the scrambled control were harvested and mixed at a concentration of 4.5 x 10^6 per ml gel. Then, 350 µl of gel mixture was pipetted in and between the Velcro attachment points. After 1 hour gelation, 3 ml PM was added which was replaced after 24 hours with DM which was refreshed daily for 4 days at which point analysis was performed.

Immunofluorescent staining

Cells were cultured on Thermanox® coverslips, Lab-Tek chamber slides or 96 wells plates (all NUNC Brand Products, Roskilde, Denmark) coated with 1 % gelatine. At 100 % confluence, cells were fixed or cultured for an additional five days in DM and subsequently fixed in 2 % paraformaldehyde (PFA) at room temperature for 10 min. A permeabilization step was performed with 0.5 % Triton X-100 (Sigma-Aldrich) in PBS at room temperature for 10 min. Non-specific binding-sites were blocked with 10 % Goat serum in PBS for 30 min. Cells were incubated with the primary antibody in PBS and 2 % serum at room temperature for 60 min or at 4 °C overnight. The primary antibody consisted of either a myogenic marker, rabbit-anti-human desmin (1:100) (Novus Biological, Littleton, USA), a fibroblast marker, mouse anti-human MCA1399G (1:100) (AbD serotec, Oxford, UK), a sarcomere component, mouse-anti-human α-sarcomeric actin IgM (1:200; clone Alpha Sr-1) (Abcam, Cambridge, UK), a myogenic transcription factor, mouse-anti-human MyoD (1:100) (Dako, Glostrup, Denmark), a sarcomere component, mouse-anti-human myosin (MF20; 1:500) and a satellite cell marker, mouse-anti-human Pax7 (1:10) (both Developmental Studies Hybridoma Bank, Iowa, USA). After three washes with 0.05 % Tween in PBS the cells were incubated with a secondary antibody-cocktail at room temperature for 30 min. The secondary antibody-cocktail constituted of FITC-conjugated goat-anti-rabbit IgG (1:100) (Southern Biotech, AL, USA), Alexa Fluor®488 goat-anti-mouse IgM and Alexa Fluor®555 goat-anti-mouse IgG1 or IgG2b (all Invitrogen) (1:300) and 10 % normal human serum in PBS/DAPI. Samples were mounted in Citifluor AP1 (Agar Scientific, Essex, UK). For Odyssey® infrared imaging (LI-COR Biosciences, Lincoln, Nebraska, US), the secondary antibody was goat-anti-mouse IrDye800 (1:500 in PBS containing DRAQ5 (1:1000) and 10 % normal human serum). Examination was performed by immunofluorescent microscopy using a Leica DMRXA microscope and Leica Software (Leica Microsystems, Wetzlar, Germany), and further quantification was performed by either TissueFAXS using a Zeiss AxioObserver.Z1 microscope and TissueQuest Cell Analysis Software (TissueGnostics, Vienna, Austria), or by Odyssey® infrared imaging system.

Bio-artificial muscle construct analysis

Contructs were washed in PBS and fixed in 10 % formalin for 1 hour. A permeabilization step was performed with 0.5 % Triton X-100 (Sigma-Aldrich) in PBS at room temperature for 30 min. Non-specific binding-sites were blocked 1 % horse serum in NET-gel twice for 20 min. Constructs were incubated with mouse-anti-human MyoD (1:100) (Dako), rabbit-anti-human desmin (1:100) (Novus Biological) and 10 %

serum in NET-gel at 4 °C overnight.
After six washes with NET-gel, the constructs were incubated with Alexa Fluor® 555 goat-anti-mouse IgG1 (1:300), FITC-conjugated goat-anti-rabbit IgG (1:100) (Southern Biotech) and 10 % normal human serum in PBS/DAPI at room temperature for 2 hours. After three washes with NET-gel, constructs were mounted between coverglasses with Mowiol. Confocal microscopy was performed using a Leica SP2 AOBS CLSM confocal microscoop (Leica Microsystems).

Gene transcript analysis

Total RNA was isolated from approximately 200,000 cells using the Rneasy Kit (Qiagen Inc., CA, USA), in accordance to the manufacturer's protocol. Briefly, a cell lysate was made and diluted with an equal volume of ethanol (70 %). RNA was collected on an RNA binding filter by centrifugation. DNA was removed by incubation with a DNase I solution at 37 °C for 15 min. The RNA-binding filter was washed twice and subsequently the RNA was eluted with 14 µl Elution Buffer. The RNA concentration and purity were determined by spectrophotometry (NanoDrop Technologies, Wilmington, NC). For qRT-PCR analysis, total RNA was reverse transcribed using the First Strand cDNA synthesis kit (Fermentas UAB, Lithuania). In summary, 1 µg of total RNA was diluted in a final reaction volume of 20 µl containing random hexamer primer (0.5 µg), RiboLock™ Ribonuclease Inhibitor (20 U), 1 mM dNTP mix, and incubated at 37 °C for 1 h. The reverse transcription reaction was terminated by heating the mixture to 70 °C for 10 min, after which the samples were placed on ice. Quantitative RT-PCR analysis was performed in a final reaction volume of 10 µl, consisting of SYBR Green Supermix (Bio-Rad, Hercules, USA), 0.5 mM primermix (Table 1) and 5 ng cDNA. For analysis of *PKT9*, Applied Biosystems 'assay on demand' primer/probe sets were used to detect amplimers of *PKT9* (Hs00702289_s1) and β-2-Microglobulin (β2M; Hs99999907_m1). Reactions were performed at 95 °C

Table 1. Primer sequence quantitative reverse transcription polymerase chain reaction.

Primer	Forward	Reverse
PAX7	ATCCGGCCCTGTGTCATCTC	CACGCGGCTAATCGAACTCA
MYOD1	AGCACTACAGCGGCGACTCC	CACGATGCTGGACAGGCAGT
ACTA1	GCCGCGATCTCACCGACTA	GCTGTTGTAGGTGGTCTCGTGAA
MYL1	AAGCCCGCAATGCAGAAGAG	TTGCTTGCAGTTTGTCCACCA
MYL3	GAACACCAAGCGTGTCATCCA	TCAGCAGATGCCAGTTTTCCA
GAPDH	CTGCCGTCTAGAAAAACCTG	GTCCAGGGGTCTTACTCCTT

for 15 s, 60 °C for 30 s, 72 °C for 30 s, for 40 cycles. Analysis of the data was per formed using Science Detection Software 2.2.2. To determine differences in expression, Ct-values were normalized against GAPDH-expression using the ΔCt-method ($\Delta Ct_{(gene)} = Ct_{(gene)} - Ct_{(GAPDH\ or\ \beta 2M)}$). Relative expression levels were calculated as $2^{-(\Delta Ct)}$. All cDNA samples were amplified in triplicate.

MicroRNA analysis

Total RNA was isolated from approximately 200,000 cells using the mirVana kit (Ambion), in accordance to the manufacturer's protocol. Briefly, a cell lysate was made and diluted with 1.25 volumes of ethanol (100 %). Total RNA was collected on a RNA binding filter by centrifugation. The RNA concentration and purity were determined by spectrophotometry (NanoDrop Technologies, Wilmington, NC). cDNA synthesis was performed using the microRNA Reverse Transcription Kit. In summary, 5 ng of total microRNA was diluted in a final reaction volume of 7.5 µl with 1.5 µl microRNA specific RT-primer mix (Table 2) and 3.5 µl RT-master mix, containing 1 mM dNTP mix, multiscribe RT enzyme, RT Buffer, RNase Inhibitor and water. This was incubated at 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min, and subsequently mixed with 2 µl microRNA specific qRT-primer mix and 10.5 µl water. Quantitative RT-PCR analysis was performed with 5 ng cDNA-primer mix and 5 µl iTaq Supermix with ROX (Bio-Rad, Hercules, USA). Reactions were performed at 95 °C for 15 s, 60 °C for 60 s, for 45 cycles. Analysis of the data was performed using Science Detection Software 2.2.2. To determine differences in expression, Ct-values were normalized against RNU6B-expression using the ΔCt-method ($\Delta Ct_{(microRNA)} = Ct_{(microRNA)} - Ct_{(RNU6B)}$). Relative expression levels were calculated as $2^{-(\Delta Ct)}$. All cDNA samples were amplified in triplicate.

Table 2. Mature microRNA sequence for qRT-PCR.

Name	Mature microRNA sequence
Hsa-miR-1	UGGAAUGUAAAGAAGUAUGUAU
Hsa-miR-206	UGGAAUGUAAGGAAGUGUGUGG
RNU6B	CGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTTT

Statistics

All data are represented as means ± SEM and were analyzed by Student's t test or analysis of variance (ANOVA) using Graph-Pad Prism Version 5 (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

The balance between quiescent satellite cells and myotubes during myogenesis

Satellite cells were isolated and cultured from enzymatically dissociated muscle tissue. Initial passages comprised of heterogeneous cell populations, but at passage 8 satellite cells had nearly reached homogeneity (Fig. 1A). Over 95 percent of the cells expressed the satellite cell marker desmin. The remaining desmin negative cells were most likely residual fibrobasts (Fig. 1B).

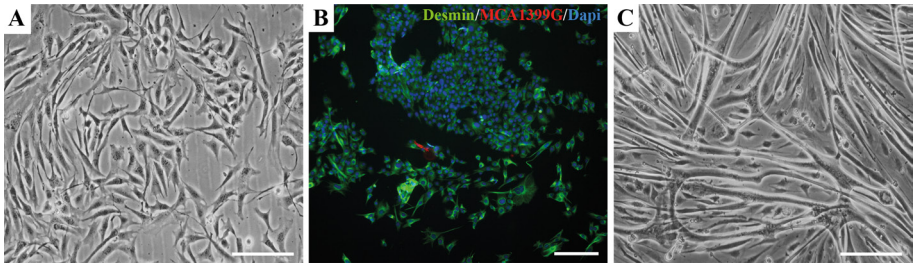


Figure 1. Satellite cell culture, differentiation and characterization. (A) During passages 8-15, cultured cells display a homogeneous morphology, note the triangular shaped cells which are typical for satellite cells, by differential interference contrast (DIC) microscopy. (B) An immunofluorescent image of proliferating cells at passage 9 double stained for a satellite cell marker Desmin (green) and a fibroblast marker MCA1399G (red). Nuclei are counterstained with DAPI (blue). (C) Over 95 percent of the cells expressed the satellite cell marker Desmin. Differentiation of confluent satellite cell cultures was induced by switching to differentiation medium. Five days after switching to differetiation medium, myotubes were clearly visible by DIC microscopy. All scale-bars are 100 µm.

Differentiation of confluent satellite cell cultures was induced by switching to differentiation medium (DM). Five days after switching to DM, part of the satellite cells had differentiated, fused and formed myotubes (Fig. 1C). Approximately 30 percent of the satellite cell remained mononucleated. These cells highly expressed Pax7. On the contrary, the nuclei of satellite cells that formed myotubes did not express Pax7. However, they expressed MyoD, and moreover, myotubes were highly positive for desmin, α-sarcomeric actin and myosin (Fig. 2A-D).

Since normally Pax7 is expressed by quiescent satellite cells, we hypothesized that satellite cell cultures which contain a relative low percentage of Pax7 expressing cells prior to differentiation, are more prone to myotube formation. Indeed, undifferentiated satellite cell cultures that showed 10.3 ± 5.6 % Pax7 expressing cells ($Pax7^{low}$), showed 61.6 ± 4.9 % more MyoD positive cells than satellite cell cultures that showed 62.3 ± 11.9 % Pax7 expressing cells ($Pax7^{high}$) (Fig. 3A-B; $p = < 0.01$). This was confirmed at gene expression level, where undifferentiated satellite cell cultures that

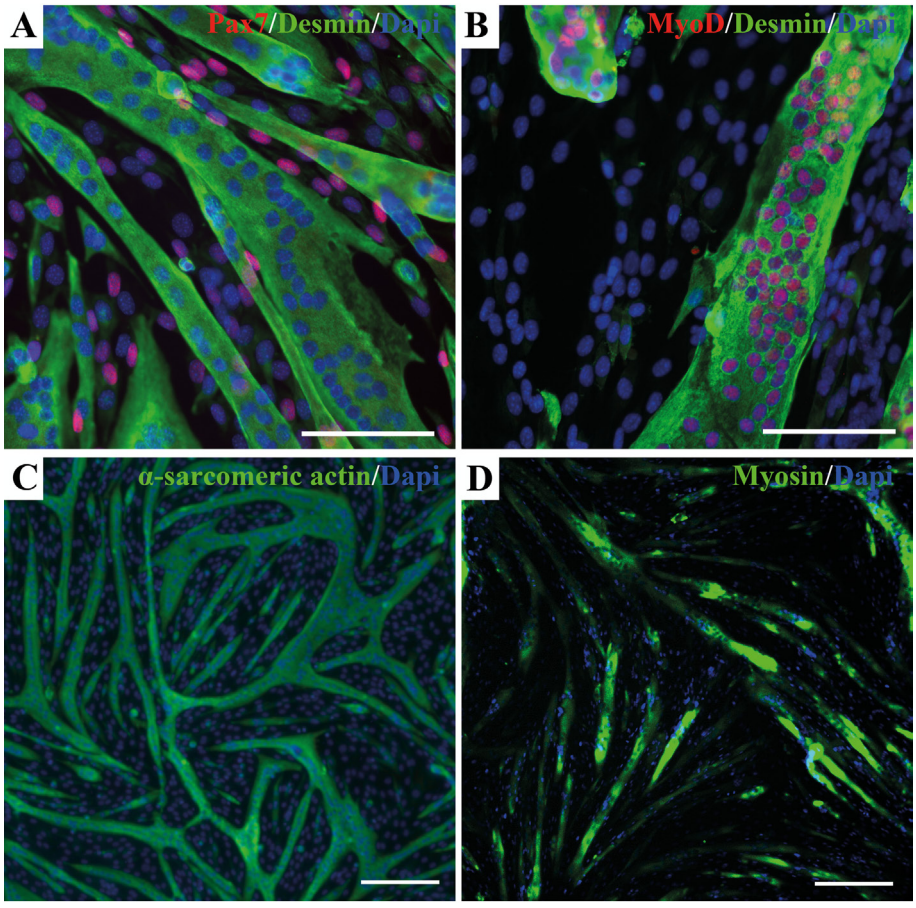


Figure 2. (A) Differentiation of confluent satellite cell cultures was induced by switching to differentiation medium. Immunofluorescent staining of differentiated satellite cell cultures after five days showed myotubes that highly expressed Desmin (green). The nuclei of quiescent satellite cells highly expressed Pax7 (red). (B) Nuclei that expressed MyoD (red) were of cells that had formed myotubes, expressing Desmin (green). Furthermore, myotubes also stained positive for (C) α-sarcomeric actin (green) and (D) Myosin (green). Nuclei are counterstained with DAPI (blue). Scalebars A-B are 50 µm and C-D are 100 µm.

expressed relative low levels of *PAX7* ($2^{-(\Delta CT)} = 0.003 \pm 0.08$) showed 43.5 ± 9.5 % higher *MYOD1* expression compared to satellite cell cultures that expressed relative high levels of *PAX7* ($2^{-(\Delta Ct)} = 0.013 \pm 0.22$) (Fig. 3C-D; $p = 0.02$).

In these undifferentiated satellite cell cultures, we measured the protein expression of the sarcomere components α-sarcomeric actin and myosin using digitalized immunofluorescent imaging (*i.e.* Odyssey®). It showed that both proteins had similar expression levels in $Pax7^{low}$ and $Pax7^{high}$ cultures. We confirmed this similar expression of α-sarcomeric actin (*ACTA1*) and myosin (*MYL1; MYL3*) at gene expression level (data not shown). However, after differentiation, α-sarcomeric actin expression was

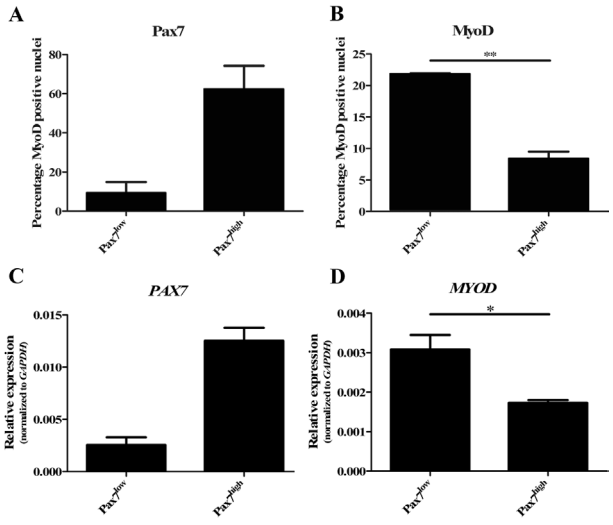


Figure 3. (A) Immunofluorescent analyses by TissueFAXS showed that there are undifferentiated satellite cell cultures that contain a relative low percentage of Pax7 expressing cells (Pax7^{low}), and cultures that contain a high percentage Pax7 expressing cells (Pax7^{high}). (B) In Pax7^{low} cultures, we showed that the percentage MyoD positive cells was 61.6 ± 4.9% higher compared to Pax7^{high} cultures (** p = < 0.01). (C) Likewise, quantitative gene expression analysis of undifferentiated satellite cell cultures showed cultures with relative low *PAX7* expression (*PAX7*^{low}), and cultures with relative high *PAX7* expression (*PAX7*^{high}). (D) Also, in *PAX7*^{low} cultures we showed 43.5 ± 9.5% higher *MYOD1* expression compared to *PAX7*^{high} cultures (* p = 0.02). (N = 4; data are represented as means ± SEM).

17.7 ± 5,4 % higher in Pax7^{low} cultures compared to Pax7^{high} cultures (Fig. 4A-B; p = 0.03). Furthermore, also at gene expression level, *ACTA1* expression was 73.6 ± 7.4 % higher in Pax7^{low} cultures (Fig. 4C; p = 0.01).

MicroRNA expression during satellite cell differentiation

We determined microRNA-1 and microRNA-206 expression in proliferation, confluent and differentiated satellite cell cultures. Using qRT-PCR, we showed that both microRNAs were upregulated 10-fold when satellite cells reached confluence. During myotube formation, microRNA-1 is upregulated an additional 100-fold, and also microRNA-206 is upregulated an additional 10-fold. The levels in which microRNA-1 and microRNA-206 were expressed, were comparable to levels we found in normal human skeletal muscle biopsies (Fig. 5A-B).

Effective satellite cell transfection with microRNA-1

Satellite cells were transfected with microRNA-1 or scrambled microRNA for forty-eight hours. We found that *PKT9* (an experimental control gene), was down-regulated by 79.5 ± 2.9 % in satellite cell cultures transfected with microRNA-1, compared to satellite cell cultures transfected with the scrambled microRNA control. This confirmed an effective cell transfection. Furthermore, we observed only a 2.0 ± 0.2 % cytotoxicity level in transfected satellite cells compared to non transfected satellite cells.

MicroRNA-1 and microRNA-206 regulate satellite cell differentiation potential

Gain-of-function approach was used to investigate the potential of microRNA-1 and microRNA-206 in human satellite cell differentiation. Transfection of undifferentiated satellite cells for forty-eight hours with microRNA-1, downregulated Pax7 protein expression by 43.3 ± 15.8 % compared to the scrambled microRNA control ($p = 0.02$). Transfection with microRNA-206 did not result in a significant decrease in Pax7 expression. After transfection for forty-eight hours, satellite cells were confluent and PM was switched to DM. Five days after switching to DM, part of the

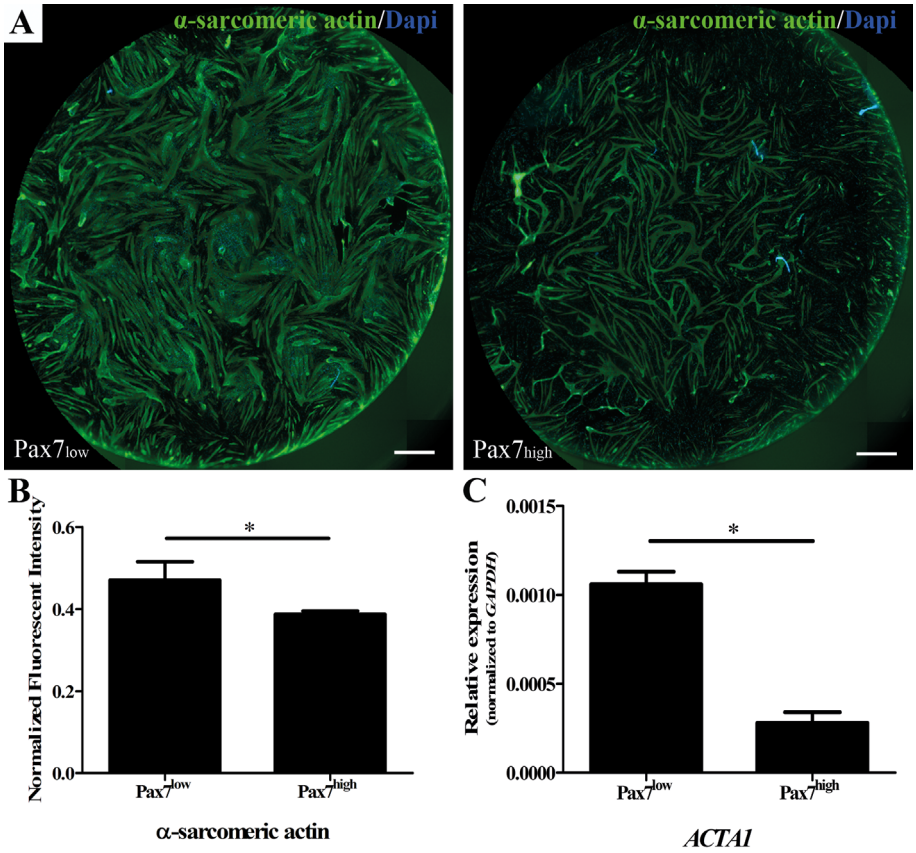


Figure 4. Differentiation of satellite cells in Pax7^{low} and Pax7^{high} cultures. (A-B) Immunofluorescent analyses of α-sarcomeric actin (green) by Odyssey, five days after switching to differentiation medium, showed that in Pax7^{low} cultures α-sarcomeric actin expression was 17.7 ± 5,4 % higher compared to Pax7^{high} cultures (* $p = 0.03$). (C) Furthermore, quantitative gene expression analysis of differentiated cultures revealed 73.6 ± 7.4 % higher *ACTA1* expression in Pax7^{low} cultures compared to Pax7^{high} cultures (* $p = 0.01$). (N = 4; data are represented as means ± SEM). Scalebars are 500 µm.

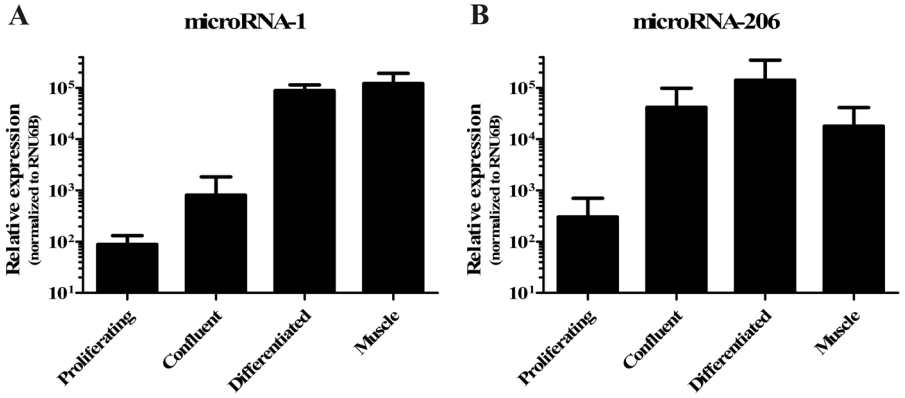


Figure 5. MicroRNA-1 and microRNA-206 expression in proliferating, confluent and differentiated satellite cells. (A-B) Quantitative microRNA expression analysis showed that both microRNAs were upregulated 10-fold in confluent satellite cells. Five days after switching to differetiation medium, microRNA-1 is upregulated 100-fold, and microRNA-206 is upregulated 10-fold. Levels in which microRNA-1 and microRNA-206 were expressed, were comparable to levels we found in normal human skeletal muscle biopsies. (N = 4; data are represented as means ± SEM).

satellite cells differentiated. In these differentiated cultures, MyoD protein expression had increased 82.0 ± 31.3 % in satellite cells transfected with microRNA-1, compared to scrambled microRNA ($p = 0.03$). MyoD expression was 59.2 ± 32.5 % increased in satellite cells transfected with microRNA-206 (Fig. 6B; $p = 0.04$). Finally, α-sarcomeric actin expression had increased 51.1 ± 24.7 % in satellite cells transfected with microRNA-1 ($p = 0.04$), and 47.9 ± 26.1 % increased in satellite cells transfected with microRNA-206 (Fig. 6C; NS). Myosin expression had increased 14.2 ± 6.2 % in satellite cells transfected with microRNA-1 ($p = 0.03$), and 32.1 ± 8.3 % increased in satellite cells transfected with microRNA-206 (Fig. 6D; $p = < 0.01$). Cross-striations were observed in differentiated satellite cells transfected with microRNA-1, microRNA-206 or the scrambled microRNA. However, no significant difference between these groups could be detected.

MicroRNA-206 increases satellite cell differentiation in a three dimensional bio-artificial muscle construct

Satellite cells transfected with microRNA-1 or microRNA-206 were subsequently cultured in a three dimensional bio-artificial muscle construct. Analysis by confocal microscopy showed that 26.7 ± 0.6 % of the nuclei in the microRNA-1 transfected muscle constructs were MyoD positive, and 31.7 ± 2.1 % of the nuclei in the microRNA-206 transfected muscle constructs were MyoD positive. Compared to cultures transfected with the scrambled microRNA control, the percentage of MyoD

expressing nuclei was increased 19.4 ± 1.2 % in cultures transfected with microRNA-1, and 31.8 ± 4.5 % in cultures transfected with microRNA-206 (Fig. 7; ** $p < 0.01$).

DISCUSSION

The current study set out to investigate whether modulation through microRNA-1 and microRNA-206 of human satellite cells could positively influence myogenesis. One major finding is that transient overexpression of microRNA-1 or microRNA-206 in satellite cells enhances differentiation potential by downregulation the satellite cell marker Pax7, thereby increasing the myogenic regulator factor MyoD and enhancing myogenic potential. Also in satellite cells cultured in a three dimensional bio-artificial muscle construct, this enhancement occurs. Moreover, sarcomere components such as α-sarcomeric actin and myosin become more abundant in microRNA transfected satellite cells.

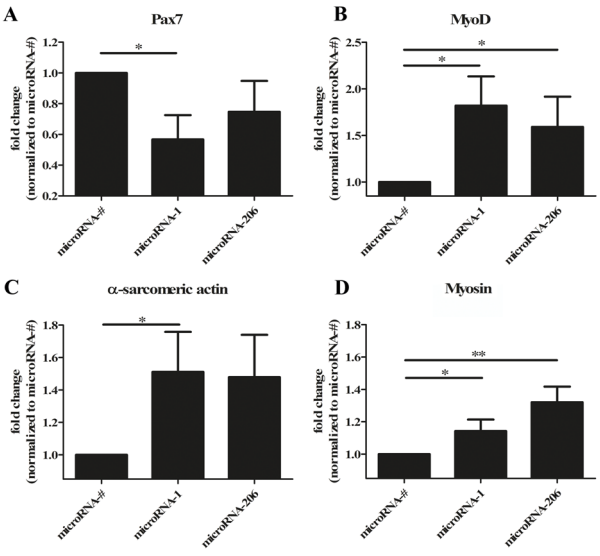


Figure 6. The effect of microRNA-1 and microRNA-206 on satellite cells. (A) Analysis by TissueFAXS showed a decrease of Pax7 expressing cells by 43.3 ± 15.8 % in proliferating satellite cell cultures transfected with microRNA-1 for forty-eight hours, compared to cultures transfected with the scrambled microRNA control (* $p = 0.02$). There was no significant decrease in the percentage of Pax7 expressing cells in cultures transfected with microRNA-206. (B) After transfection for forty-eight hours, satellite cells reached confluence and differentiation was initiated by swiching to differentiation medium for five days. In these differentiated cultures, the percentage MyoD expressing cells was increased 82.0 ± 31.3 % in cultures transfected with microRNA-1, and increased 59.2 ± 32.5% in cultures transfected with microRNA-206 compared to cultures transfected with the scrambled microRNA control (* $p = 0.03$ and $p = 0.04$ respectively). (C) Immunofluorescent analysis by Odyssey of α-sarcomeric actin showed an increase of 51.1 ± 24.7 % in cultures transfected with microRNA-1 compared to cultures transfected with the scrambled microRNA control (* $p = 0.04$). There was no significant increase in α-sarcomeric actin expression in cultures transfected with microRNA-206. (D) Myosin expression increased 14.2 ± 6.2 % in cultures transfected with microRNA-1, and increased 32.1 ± 8.3 % in cultures transfected with microRNA-206 compared to cultures transfected with the scrambled microRNA control (* $p = 0.03$ and ** $p = < 0.01$ respectively). (N = 4; data are represented as means ± SEM).

Our finding that when Pax7 expression is low in proliferating satellite cells, MyoD expression is high, implies that these cells are geared up for myogenic differentiation *i.e.* myotube formation. This results in an increased upregulation of the sarcomere component α-sarcomeric actin, and more efficient myotube formation during differentiation. This differentiation process is caused by activation of the myogenic regulator factors MyoD and Myf5, after which Pax7, a transcription factor that inhibits differentiation and maintains quiescent satellite cell state, is downregulated [145,146].
Our results show that microRNA-1 and microRNA-206 are highly upregulated during differentiation of human satellite cells. Through transient transfection of human satellite cells with microRNA-1 and microRNA-206, we show that microRNA-1 correlates with a downregulation of Pax7, resulting in an upregulation of MyoD and subsequently, a higher α-sarcomeric actin and myosin expression. The explanation for this increased differentiation is that microRNA-1 and microRNA-206 are transcribed simultaneously with *MYOD1*, which is upregulated during differentiation [103,137]. MicroRNA-1 and microRNA-206 bind to the 3'UTR of Pax3, thereby downregulating Pax3. Since Pax3 is responsible for preserving quiescent satellite cell state and preventing differentiation, downregulating Pax3 promotes differentiation of satellite cells [103]. Furthermore, microRNA-1 and microRNA-206 also bind to the 3'UTR of Pax7, downregulating Pax7 and even further promoting differentiation in satellite cells [102,104]. Although we did not find a significant downregulation of Pax7 upon transfection with microRNA-206, satellite cells did show a significant upregulation of MyoD, and also a higher α-sarcomeric actin and myosin expression. This discrepancy in microRNA-206 regulation of Pax7 might be caused by differences, such as rodent versus human or cell line versus primary cells [147,148], which makes translation to primary human cells difficult. Furthermore, heterogeneity with respect to function, behavior and different subsets within the satellite cell population [43,135,139,149,150] might be responsible for this discrepancy. The heterogeneity is caused by different capacities of satellite cells; they are capable of regenerating muscle fibers and meanwhile replenishing their own pool of progenitor cells [90,91]. On the other hand, these qualities of simultaneous regeneration and self renewal render satellite cells highly suitable for tissue engineering.
Furthermore, transient transfection with microRNA-206 alone may not be sufficient to downregulate Pax7 significantly, but it might be possible that a slight downregulation at an earlier timepoint, is sufficient to influence downstream effects, such as upregulating MyoD, α-sarcomeric actin and myosin. Also, in recently published studies, microRNA-1 and microRNA-206 are co-administered to repress Pax7 [102],

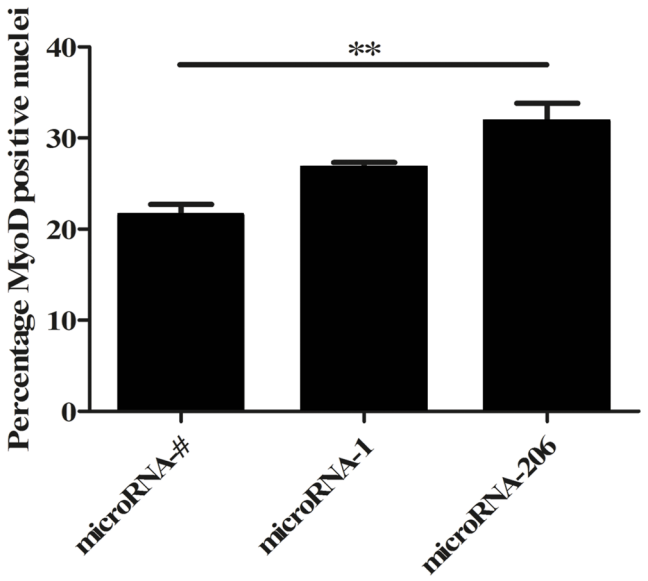


Figure 7. The effect of microRNA-1 and microRNA-206 on satellite cells in a three dimensional bio-artificial muscle construct. The percentage of MyoD expressing cells increased 19.4 ± 1.2 % in cultures transfected with microRNA-1, and 31.8 ± 4.5 % in cultures transfected with microRNA-206 compared to cultures transfected with the scrambled microRNA control (** $p < 0.01$). (N = 3; data are represented as means ± SEM).

which may indicate that transfection with both microRNAs is necessary to have a significant effect.

Remarkably, we were unable to show a loss-of-function after transient transfection with anti-microRNA antisense molecules specific for the mature microRNA-1 and microRNA-206 sequence (anti-miR for hsa-miR-1, AM10617; anti-miR for hsa-miR-206, AM10409, Ambion). The expression of Pax7, MyoD, α-sarcomeric actin or myosin did not change significantly in either proliferating satellite cell cultures or differentiated satellite cell cultures (data not shown). Most likely, the antisense molecules cannot be offered in sufficient amounts to reach the threshold necessary to exert a functional or measurable effect as a result of the high increase in endogenous microRNA-1 and microRNA-206 in differentiating human satellite cells, and the transient character of transfection in our study.

Our results demonstrate that microRNA-1 and microRNA-206 promote satellite cell differentiation through a downregulation of Pax7 and/or an upregulation of MyoD. However, transfection with either microRNA-1 or microRNA-206 solely, was not sufficient to trigger myotube formation in proliferating or even confluent satellite cells. For that to occur, confluency and switching to differentiation medium was still required. Thus, microRNA-1 and microRNA-206 enhance muscle differentiation, but require other mediators to initiate myotube formation.

Several microRNAs have been identified that are involved in skeletal muscle proli-

feration and differentiation [94,95]. Besides the pronounced role for microRNA-1 and microRNA-206, there is involvement of microRNA-27. Through downregulating Pax3, microRNA-27 forces satellite cells to start differentiation [109]. Furthermore, for tissue engineering applications, transfection with microRNAs offers a novel tool for modulating efficient cell function. MicroRNA-133 is involved in maintaining proliferation of satellite cells through repressing Serum Response Factor. The inhibition of microRNA-133 in murine satellite cells decreased their proliferation [100,148]. In a three dimensional model, *i.e.* a bio-artificial muscle [144,148], this inhibition of microRNA-133 improved expression of a differentiation marker Mef2 and moreover and improved contractile force [143]. Upon culture in a three dimensional bio-artificial muscle construct, satellite cells transfected with microRNA-206 showed an increased MyoD expression. Therefore, we conclude that in a scaffold consisting of extracellular matrix microRNA-206 improves the generation of myotubes. After confirming the augmenting effect of microRNAs in two dimensional cultures thoroughly, we show that this also occurs in a three dimensional set-up, paving the way for future functional *in vitro* and *in vivo* experiments.

We have shown that microRNA-1 and microRNA-206 improve differentiation of human satellite cells. However, in optimizing differentiation, it is key to prevent complete depletion of the pool of quiescent satellite cells, known as reserve cells [151]. This population of satellite cells per myofiber naturally decreases with increasing age *in vivo*, as does their myogenic capacity [131,132,152]. Therefore, to promote tissue engineering of skeletal muscle from autologous satellite cells novel approaches such as microRNAs contribute to improve tissue engineering of human skeletal muscle for clinical application. Furthermore, for *in vivo* implantation, preservation of a pool of progenitor cells and their regenerative capacity in the tissue engineered construct is important. Transient transfection is a good option, for it modulates satellite cells in entering the differentiation program, without being a complete knockdown of quiescent satellite cell preserving factors. It merely provides a kick start in differentiation of satellite cells during tissue engineering of skeletal muscle. Maintaining the balance between genetic regulatory transcription factors and epigenetic regulatory microRNAs is vital. Therefore, in future studies, we aim to identify players that are responsible for the balance between regeneration of skeletal muscle and quiescence of satellite cells.

In conclusion, we show that microRNA-1 and microRNA-206 improve human satellite cell differentiation potential. And this represents a novel approach for tissue engineering of human skeletal muscle for the benefit of patients with facial paralysis.

CHAPTER

5

HYPOXIA PROMOTES PROLIFERATION OF HUMAN MYOGENIC SATELLITE CELLS: A POTENTIAL BENEFACTOR IN TISSUE ENGINEERING OF SKELETAL MUSCLE.

Merel Koning, Paul M.N. Werker, Marja J.A. van Luyn, Martin C. Harmsen

TISSUE ENG PART A. 2011 JUL;17(13-14):1747-58

ABSTRACT

Facial paralysis is a physically, psychologically and socially disabling condition. Innovative treatment strategies based on regenerative medicine, in particular tissue engineering of skeletal muscle, are promising for treatment of patients with facial paralysis.

The natural source for tissue engineered muscle would be muscle stem cells, i.e human satellite cells (SC). *In vivo*, SC respond to hypoxic, ischemic muscle damage by activation, proliferation, differentiation to myotubes and maturation to muscle fibers, while maintaining their reserve pool of SC. Therefore, our hypothesis is that hypoxia improves proliferation and differentiation of SC. During tissue engineering a 3D construct, or implanting SC *in vivo*, SC will encounter hypoxic environments. Thus, we set out to test our hypothesis on SC *in vitro.* During the first five passages hypoxically cultured SC proliferated faster than their counterparts under normoxia. Moreover, also at higher passages, a switch from normoxia to hypoxia enhanced proliferation of SC. Hypoxia did not affect the expression of SC markers desmin and *NCAM*. Yet, the average surface expression per cell of *NCAM* was downregulated by hypoxia and it also downregulated the gene expression of *NCAM*. The gene expression of the myogenic transcription factors *PAX7, MYF5* and *MYOD1* were upregulated by hypoxia. Moreover, gene expression of structural proteins α-sarcomeric actin (*ACTA1*), and myosins *MYL1* and *MYL3* was upregulated by hypoxia during differentiation. This indicates that hypoxia promotes a pro-myogenic shift in SC. Finally, Pax7 expression was not influenced by hypoxia and maintained in a subset of mononucleated cells, while these cells were devoid of structural muscle proteins. This suggests that during myogenesis *in vitro*, at least part of the SC adopt a quiescent, *i.e.* reserve cells, phenotype. In conclusion, tissue engineering under hypoxic conditions would seem favorable in terms of myogenic proliferation, while maintaining the quiescent SC pool.

INTRODUCTION

Paralysis of the facial muscles is a physical disabling condition which, due to impaired facial expression, leads to significant physical, psychological and social distress. Despite advancing knowledge and improved surgical techniques, outcome of conventional treatment strategies improve the quality of life of patients but genuine regeneration is not achieved [4,12,153]. The human face holds 23 paired facial muscles and one unpaired, the orbicularis oris muscle [1]. With autologous muscle transplantations, only one or two muscles can be reanimated, subtlety in movement can't be achieved fully, and patients are often hindered by autostatic syndrome [5]. Innovative treatment strategies are warranted, such as regenerative medicine. In particular tissue engineering of skeletal muscle is promising for treatment of patients with facial paralysis. Engineered facial muscles may be customized to the needs of the individual patient which will resolve the patients' physical symptoms and restore near to normal mimical function.

To accomplish this, a 3 dimensional construct must be engineered consisting of functional skeletal muscle fibers, vasculature and innervation. Combining these premises to a clinically functional construct remains a major challenge. For vascularisation, 4 main techniques are currently subject of investigation: First, through *in vivo* implantation of a preformed 3 dimensional muscle construct around a vascular pedicle [34]. Second, through implantation of myoblasts in a prevascularised fibrin gel [37]. Third, through stacking sheets of myoblasts upon a vascular bed, the so called cell sheet method [78,80]. And finally through co-culturing myoblasts with endothelial cells [25]. To innervate a muscle construct, it is necessary to create a functional neuromuscular junction. This could be reached through co-culturing myoblasts and neural tissue [72], and by means of electrical stimulation [35].

Muscle tissue has its own endogenous repair and maintenance system which is based on myogenic progenitor cells, *i.e.* satellite cells. The regenerative capacities and myogenic characteristics of these satellite cells has been topic of many studies that aim to use satellite cells to tissue engineer muscle [85,130,154]. In response to hypoxic ischemia in skeletal muscle, e.g. after exercise or injury, satellite cells are activated. These activated satellite cells start to proliferate and differentiate, which contributes to regeneration of the affected muscle [82,84,145].

In tissue engineering functional skeletal muscle, angiogenesis and neurogenesis are imperative. However, vascularisation of a tissue engineered muscle construct may not be instantly functional after implantation *in vivo*. Thus, satellite cells in the center of

the muscle construct will encounter hypoxia. Therefore, in this study, we focus on the influence of hypoxia on myogenesis *in vitro*.

In general, hypoxia is a crucial limiting factor in tissue engineering of constructs that exceed approximately 0.2 mm thickness. In these constructs a steep oxygen concentration gradient occurs, with hypoxic levels inside which may result in cell death after implantation [155]. *In vivo*, skeletal muscle is shown to adapt to a prolonged hypoxic environment through upregulating myostatin, causing atrophy [156]. In mesenchymal stem cells however, hypoxic- preconditioning *in vitro* had profound benefits for cell survival after implantation [157]. Bovine satellite cells were shown to both proliferate and differentiate more efficiently under hypoxia [158]. Yet, species differences may exist, and the influence of hypoxia on human satellite cells was not fully investigated yet. The importance of *in vitro* research shows from a resent clinical trial where myoblasts, *i.e.* satellite cells, were transplanted into a hypoxic environment of the ischemic heart of patients suffering from heart failure. However, echocardiographic heart function did not improve and arrhythmia occurred, indicating cellular dysfunction [159]. In tissue engineering, as in cell therapy, satellite cells will encounter hypoxic environments. Therefore, for clinical application of human satellite cells, a better understanding of the response of human satellite cells to changes in oxygen level is required. In tissue engineering facial muscles, although they are thinner than other skeletal muscles, their thickness exceeds 0.2 mm. Hence, satellite cells encounter lower oxygen concentrations. Therefore, before applying these muscle constructs *in vivo*, we need to study how satellite cells react to a hypoxic environment. As satellite cells *in vivo* appear to respond to hypoxic muscle damage with activation, proliferation, differentiation and maturation, while maintaining their reserve pool of satellite cells, our hypothesis is that *in vitro* hypoxia improves proliferation and differentiation of human satellite cells.

MATERIALS AND METHODS

Satellite cell isolation

Muscle biopsies were obtained from 8 healthy human donors, 30 to 66 years of age, undergoing reconstructive surgery. The age of the donors was 51.7 ± 10.6 years, and there were 3 males and 5 females included. The study protocol was approved by our institutional ethics committee, and patients gave their informed consent. A muscle biopsy of either the latissimus dorsi (3 donors) or the orbicularis oculi muscle (5 donors)

was collected in cold (4 °C) phosphate buffered saline (PBS) supplemented with 10 % penicillin/streptomycin 50 µg/ml (Sigma-Aldrich, St. Louis, USA). Specimens were stripped of any visible connective tissue and fat, weighted and minced. The muscle specimen weight ranged between 200 mg and 800 mg. Enzymatic digestion was performed in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen/Gibco, CA, USA) containing 0.04 mg/ ml (0.16 Collagenase Wünsch units/ml) Liberase Blendzyme 3 (Roche Applied Science, the Netherlands) at 37 °C for 1 hour. The suspension was filtered through a 70 µm filter, 10 % Fetal Bovine Serum (FBS; Invitrogen/Gibco) was added to the flow through and centrifuged at 300xg for 10 min at 4 °C after which the supernatant was discarded. Lysis-buffer was used to eliminate erythrocytes. After 2 min on ice, the suspension was centrifuged again at 300xg for 10 min at 4 °C after which the supernatant was discarded and the pellet gently resuspended in proliferating medium (PM), containing DMEM, 20 % FBS, and 1 % penicillin/streptomycin.
Undigested tissue was subjected to another 2-3 cycles of digestion process. The different cell suspensions were pooled and viable cells were counted using the trypan blue exclusion method with a Bürker counting chamber.

Satellite cell culture

Immediately after isolation, the cell suspension was divided into two fractions and cultured either under normoxia (21 % O_2) or hypoxia (2 % O_2) at 37 °C. Cells were plated at 5.0 x 10^3 cells/cm^2 on culture flasks precoated with 1 % gelatine. Medium was refreshed three times per week.
When cells reached 70 % confluence they, were enzymatically harvested using accutase (Invitrogen), counted with a Coulter Counter and replated at 5.0 x 10^3 cells/cm^2 on 1 % gelatine precoated flasks.
Myogenic differentiation of satellite cells was induced at 100 % confluence by switching to differentiation medium (DM), containing DMEM, 2 % FBS, 1 % penicillin/streptomycin, 1 % Insulin-Transferrin-Selenium-A (100x) (Invitrogen) and 0.4 µg/ml dexamethason (Sigma-Aldrich).
Passage number (P_x) was defined as the xth sequential harvest of a subconfluent cell population. All differentiation experiments were performed using P6 - P15.
The population doubling time was calculated $N(t) = C(2)^{\wedge t/d}$, N(t) = the number of cells at time t d = doubling period C = initial number of cells t = time (days).
A CyQUANT cell proliferation assay (Invitrogen) was performed in accordance to the manufacturer's protocol to determine proliferation.

Immunofluorescent staining

Cells were cultured in PM on Thermanox® coverslips (NUNC Brand Products, Roskilde, Denmark) coated with 1 % gelatine in a 24 wells plate. At 100 % confluence, cells were washed twice with PBS and either cultured an additional five days in DM, or fixed in 2 % paraformaldehyde (PFA) at room temperature for 10 min. Cells were dried under a ventilator and stored -20 °C. After thawing, a permeabilization step was performed with 0.5 % Triton X-100 (Sigma-Aldrich) in PBS at room temperature for 10 min. Non-specific binding-sites were blocked with 10 % Goat serum in PBS for 30 min. Cells were incubated with the primary antibody in PBS and 2 % Goat serum at room temperature for 60 min. The primary antibody consists of either a myogenic marker, rabbit-anti-human desmin (1:100) (Novus Biological, Littleton, USA), a proliferation marker, mouse-anti-human Phosphohistone H3 (1:500) (clone 3H10; Millipore International Inc., Temecula, CA, USA), a satellite cell marker, mouse-anti-human NCAM (1:200) (clone MEM-188; Biolegend, San Diago, CA, USA), a fibroblast marker, mouse anti-human MCA1399G (1:100) (AbD serotec, Oxford, UK) an adult muscle marker, mouse-anti-human myosin (MF20; 1:500) and a satellite cell marker, mouse-anti-human Pax7 (1:10) (both Developmental Studies Hybridoma Bank, Iowa, USA). After three washes with 0.05 % Tween in PBS the cells were incubated with a secondary antibody-cocktail at room temperature for 30min. The secondary antibody-cocktail was constituted of FITC-conjungated goat-anti-rabbit IgG (Southern Biotech, AL, USA), Texas RedtmX-conjungated donkey-anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA), Alexa Fluor® 555 goat-anti-mouse IgG1 or IgG2b (both Invitrogen) (all 1:200 in PBS/DAPI containing 10 % normal human serum). After 3 washes with PBS the slides were mounted in Citifluor AP1 (Agar Scientific, Essex, UK). Examination was performed by immunofluorescent microscopy using a Leica DMRXA microscope and Leica Software (Leica Microsystems, Wetzlar, Germany), and further quantification was performed by TissueFAXS using a Zeiss AxioObserver.Z1 microscope and TissueQuest Cell Analysis Software (TissueGnostics, Vienna, Austria).

Furthermore, for flow cytometry approximately $5x10^5$ cells were resuspended in 100 ml FACS buffer consisting of PBS (4 salts), 2 mM EDTA and 0.5 % (v/v) FBS. Cells were incubated with 5 µl mouse monoclonal FITC-conjugated antibody anti-NCAM (Biolegend, San Diego, CA, USA) at 4 °C for 30 min. After two washes with FACS buffer, cells were resuspended in 300 µl FACS buffer and examined by FACS analysis on a FACSCaliber system (BD Biosciences, San Jose, CA). The analysis was performed using WinList™ 6.0 software (Verity Software House).

Gene transcript analysis

At day 0 and day 5, after switching to DM, total RNA was isolated from approximately 200,000 cells using the Rneasy Kit (Qiagen Inc., CA, USA), in accordance to the manufacturers protocol. Briefly, a lysate was made and diluted with an equal volume of ethanol (70 %). RNA was collected on an RNA binding filter by centrifugation. DNase treatment was performed by incubation with a DNase I solution at 37 °C for 15 min. The RNA-binding filter was washed twice and subsequently the RNA was eluted with 14 µl Elution Buffer. The RNA concentration and purity were determined by spectrophotometry (NanoDrop Technologies, Wilmington, NC). For RT-PCR ana lysis, total RNA was reverse transcribed using the First Strand cDNA synthesis kit (Fermentas UAB, Lithuania). In summary, 1 µg of total RNA was diluted in a final reaction volume of 20 µl containing random hexamer primer (0.5 µg), RiboLock™ Ribonuclease Inhibitor (20 U), 1mM dNTP mix, and incubated at 37 °C for 1 h. The reverse transcription reaction was terminated by heating the mixture to 70 °C for 10 min, after which the samples were placed on ice. RT-PCR was performed in a final reaction volume of 25 µl, consisting of 10x Taq polymerase buffer, 0.25 mM dNTP mix, 1.5 mM MgCl2, 1 µl primer-mix (Table 1) and 1 U Taq DNA Polymerase.

Table 1. Primer sequence qualitative RT-PCR.

Primer	Forward	Reverse
PAX7	GATTTTTGCCGACTTTGGAT	GTGGGCCATCTCCACTATCT
MYF5	AACCCTCAAGAGGTGTACCA	GTGATCCGGTCCACTATGTT
MYOD1	AGCACTACAGCGGCGACT	CGACTCAGAAGGCACGTC
NCAM	GGATCTCAGTGGTGTGGAAT	TTTGTGTTTCCAGATGATGG
DES	CATCGCGGCTAAGAACATTT	TGTTGTCCTGGTAGCCACTG
ACTA1	GCCGCGATCTCACCGACTA	GCTGTTGTAGGTGGTCTCGTGAA
GAPDH	CTGCCGTCTAGAAAAACCTG	GTCCAGGGGTCTTACTCCTT

PCR reactions were performed at 94 °C for 45 s, 57 °C for 45 s, 72 °C for 1 min, for 35 cycles. Amplimers were separated in a 2 % agarose gel and stained with 0.5 µg/ml ethidium bromide after electrophoresis.

Quantitative RT-PCR analysis was performed in a final reaction volume of 10 µl, consisting of 4.5 µl SYBR Green Supermix (Bio-Rad, Hercules, USA), 0.5 µl primer-mix (0.5 mM) (Table 2) and 5 µl cDNA (1 ng/µl). Reactions were performed at 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s, for 40 cycles. Analysis of the data was performed using Science Detection Software 2.2.2.

Table 2. Primer sequence quantitative RT-PCR.

Primer	Forward	Reverse
PAX7	ATCCGGCCCTGTGTCATCTC	CACGCGGCTAATCGAACTCA
MYF5	CTATAGCCTGCCGGGACA	TGGACCAGACAGGACTGTTACAT
MYOD1	AGCACTACAGCGGCGACTCC	CACGATGCTGGACAGGCAGT
NCAM	TGCGAGGTATTTGCCTATCC	GTCGATGGATGGTGAAGAGG
DES	CATCGCGGCTAAGAACATTT	TGTTGTCCTGGTAGCCACTG
ACTA1	GCCGCGATCTCACCGACTA	GCTGTTGTAGGTGGTCTCGTGAA
MYL1	AAGCCCGCAATGCAGAAGAG	TTGCTTGCAGTTTGTCCACCA
MYL3	GAACACCAAGCGTGTCATCCA	TCAGCAGATGCCAGTTTTCCA
GAPDH	CTGCCGTCTAGAAAAACCTG	GTCCAGGGGTCTTACTCCTT

Statistics

All data are represented as means ± SEM and were analyzed by Student's t test or analysis of variance (ANOVA) using Graph-Pad Prism Version 5 (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

Proliferation of cells is initially increased under hypoxia

Cells isolated from enzymatically dissociated muscle tissue were divided into two fractions, one of which was cultured under normoxia (21 % O_2), while the other fraction was cultured under hypoxia (2 % O_2) during all passages. On average 1.25 ± 0.25 x 10^5 viable cells were isolated per gram of muscle tissue. We observed that the morphology of adherent cells was independent of the oxygen level. During low passages (P0-5), cultures under both oxygen conditions contained both spindle-shaped and typical triangular 'satellite-like' cells. During these low passages, the cells reached confluency twice as fast under hypoxia than under normoxia. The population doubling time (PDT) at passage 0-5, determined by cell count, was 8,3 days for normoxic and 4.3 for hypoxic cultures ($p = 0.04$, Fig. 1A).

At passage 6-8, in both oxygen conditions, colony forming units (CFU) appeared and the cell population became homogeneous, *i.e.* only the 'triangular' shaped cell types had remained. This occurred one or two passages later in normoxic than in hypoxic cultures. The PDT of cells at high passage (P6-15) decreased 3 to 7 fold and became similar for both oxygen conditions, namely 1.3 for normoxic and 1.2 for hypoxic cultures (Fig. 1B).

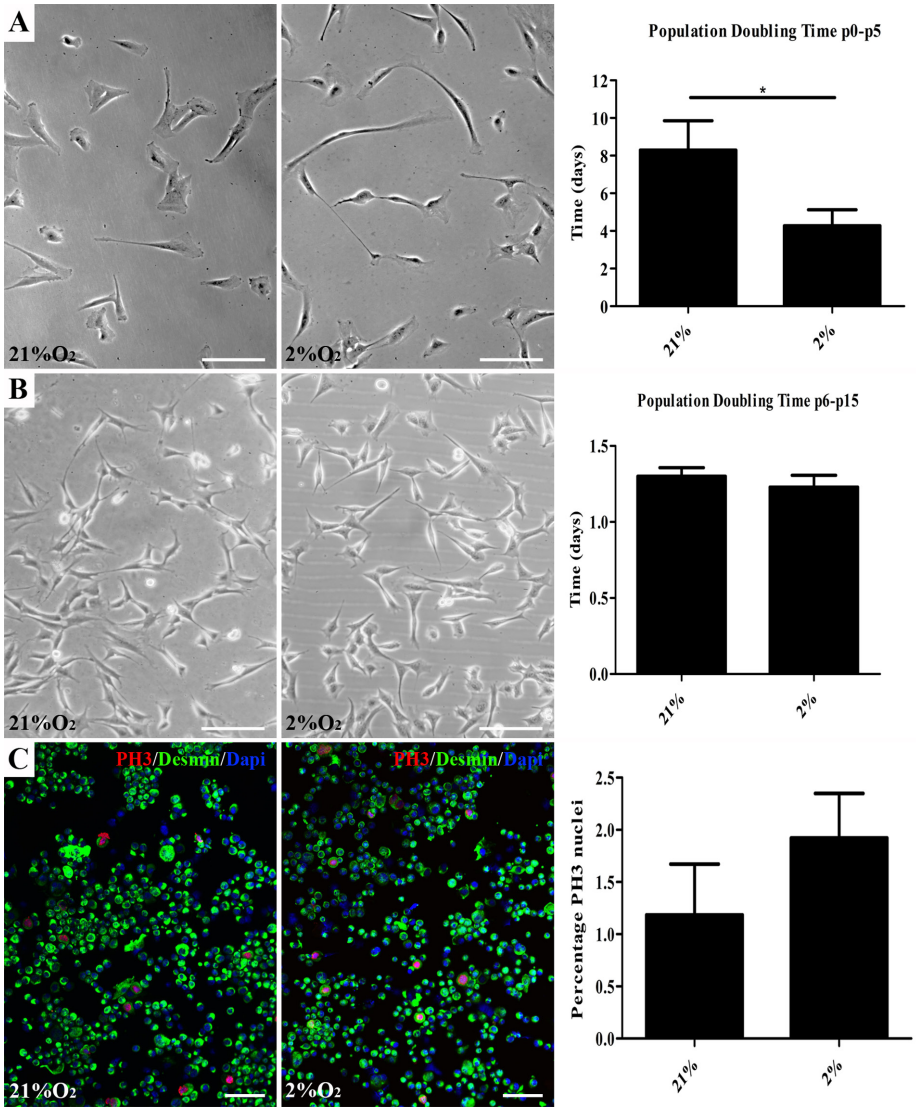


Figure 1. Morphology and Proliferation. (A) During passages (P) 0-5, adhered cells had a heterogeneous morphology both at hypoxic and normoxic culture conditions. The population doubling time (PDT) was lower for hypoxic cultures (* p = 0.04). (B) During P6-P15, the cultured cells adopted a homogeneous morphology, note the triangular shaped cells which are typical for satellite cells. At P6-P15 the PDT decreased, and became comparable for both oxygen levels. (C) This was further corroborated by similar staining patterns for phospho-histone H3 (proliferation, red), Desmin (satellite cells, green) at P6- P15. Nuclei are counterstained with DAPI (blue). Quantification of phosphohistone H3 positive cells at P6-P15 by TissueFAXS showed no significant difference in proliferation between hypoxic and normoxic culture conditions. (N = 7; data are represented as means ± SEM) All scalebars are 100 µm.

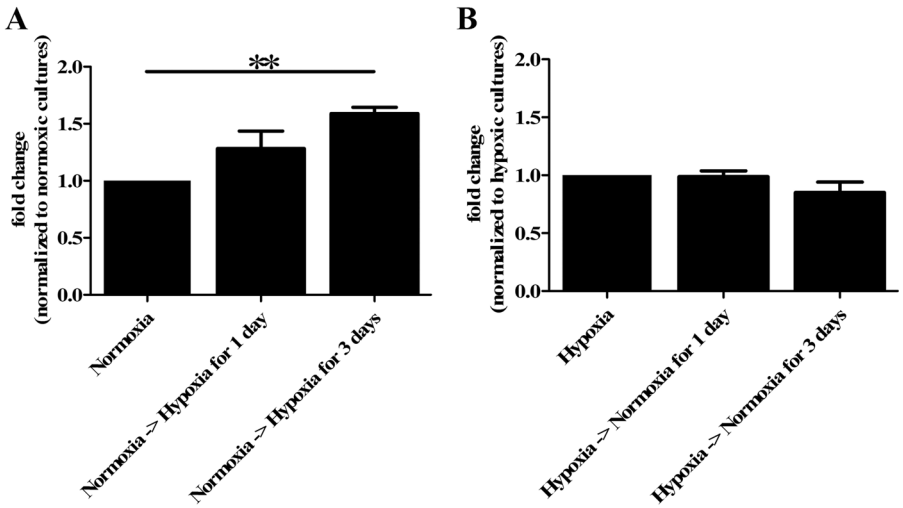


Figure 2. Proliferation after switching oxygen concentration. (A) The CyQUANT assay showed an increased proliferation when cells at high passage (P6-P15) were transferred from normoxia to hypoxia. Hypoxic culture conditions significantly stimulated satellite cell proliferation rate by day 3 compared to satellite cells that remained in normoxic culture conditions (** p = 0.01). (B) In contrast, the proliferation of cells transferred from hypoxia to normoxia remained unchanged. (N = 7; data are represented as means ± SEM).

Quantification by TissueFAXS analysis after staining of phosphohistone H3, a marker for proliferation, confirmed the lack of significant difference in proliferation of high passage cells under normoxia or hypoxia (Fig. 1C).

The CyQUANT assay also showed no significant difference in proliferation rate between cells under normoxia or hypoxia at high passage. However, *in vivo*, hypoxic insults actually represent a shift from normoxia to hypoxia, which cause satellite cells to respond either directly or indirectly, with proliferation. We investigated the influence of a decrease or increase of oxygen concentration on satellite cells that we had cultured under normoxic or hypoxic conditions respectively. Remarkably, the transfer of cells from normoxic to hypoxic culture conditions, significantly increased their proliferation, with a maximum at 3 days (p = 0.01, Fig. 2).

Desmin and NCAM expression by satellite cells is independent of the oxygen level

To further qualify and to quantify satellite cells in our cultures, the expression of desmin, a myogenic cytoskeletal protein, was determined by immunofluorescent staining. Of the cells cultured under normoxia 59.8 ± 13.7 % expressed desmin, and of the cells from the same donor, cultured under hypoxia this was 41.1 ± 18.4 % (Fig. 3B).

This is lower, but does not differ significantly.

The expression of NCAM, which is frequently considered a satellite cell marker, was measured by flow cytometry. Only approximately one third (28.4 ± 10.3 %) of the satellite cells cultured under normoxia expressed NCAM, while no more than one fifth (18.2 ± 6.5 %) of the cells cultured under hypoxia expressed NCAM. The mean fluorescent intensity (MFI) of NCAM positive cells in normoxic cultures (27.1 ± 6.6) was significantly higher than in hypoxic cultures (11.6 ± 2.0, p = 0.04, Fig. 3C-D).

Together, these findings support that desmin expression by satellite cells is unaffected by the oxygen level these cells are cultured in. Though the percentage of NCAM expressing cells is not affected, the NCAM expression per cell is decreased when satellite cells are cultured under hypoxia.

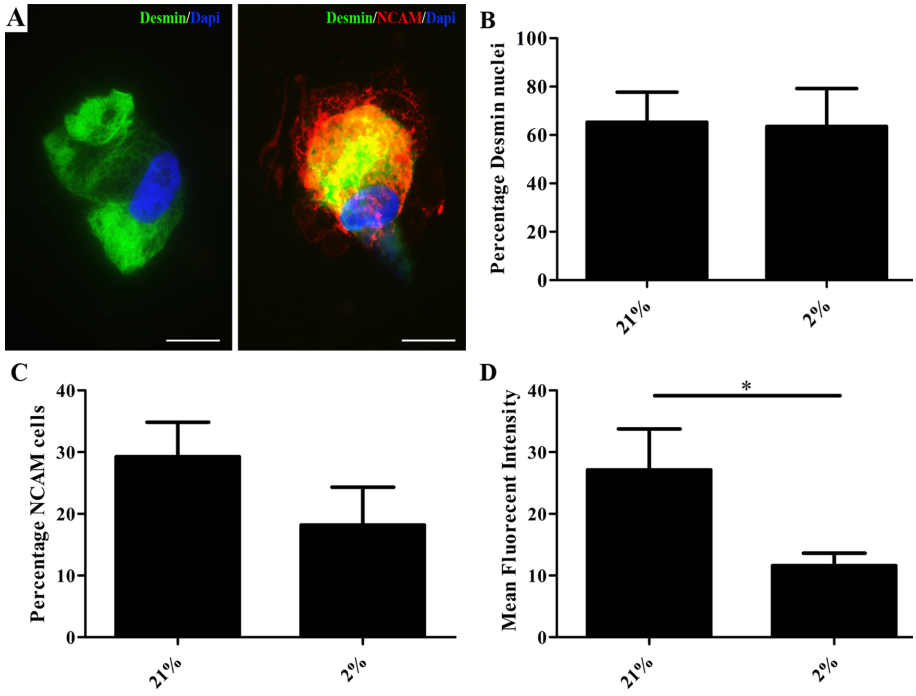


Figure 3. Protein expression analyses. (A) Immunofluorescent image of a proliferating satellite cell at passage 3 showing positive staining for Desmin (green) and a proliferating satellite cell at passage 3 showing double positive staining for Desmin/NCAM (green/red). Nuclei are counterstained with DAPI (blue). (B-C) Quantification of satellite cells during passage 3-15 showed that the fraction of either Desmin expressing cells or NCAM expressing cells did not differ between hypoxic and normoxic culture conditions. (D) However, the Mean Fluorescent Intensity (MFI), measured by FACS, of NCAM expression was significantly higher in proliferating cells cultured under normoxia than hypoxia (* p = 0.04). (N = 8; data are represented as means ± SEM) All scalebars are 20 µm.

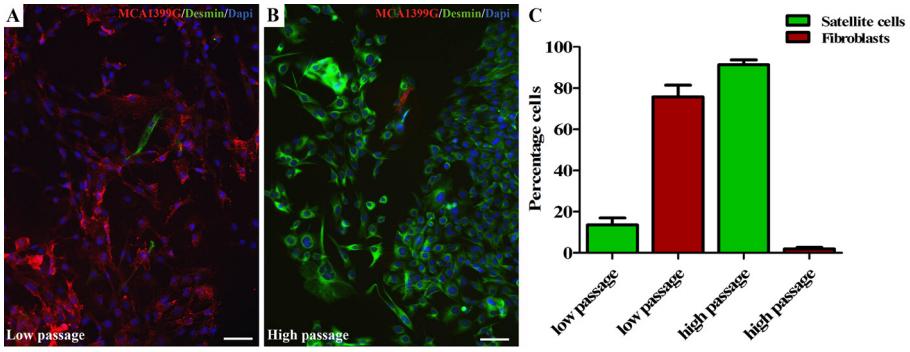


Figure 4. Satellite cell purity and fibroblast contamination. Immunofluorescent image of proliferation cell cultures at (A) low passage (P3-P4) and (B) high passage (P9-P10) double stained for a satellite cell marker Desmin (green) and a fibroblast marker MCA1399G (red). Nuclei are counterstained with DAPI (blue). (C) Quantification by TissueFAXS shows that in low passage the percentage satellite cells is low, while the percentage of fibroblasts is high. In high passage, the percentage of satellite cells is high, while the percentage of fibroblasts is low. Also, there was no effect of hypoxia on satallite cell purity or fibroblast contamination. (N = 4; data are represented as means ± SEM) Scalebars are 100 µm.

High satellite cell purity in high passage cultures is not influenced by hypoxia

To determine fibroblast contamination in our cell population, we performed a double staining of cells in low passage and in high passage with desmin and a fibroblast marker MCA1399G, and quantified with the TissueFAXS. In low passage cultures we found 13.5 ± 9.3 % desmin positive cells and 75.6 ± 5.8 % fibroblasts (Fig. 4A-C). In high passage cultures, we found 91.3 ± 4.1 % desmin positive cells and 1.9 ± 0.7 % fibroblasts (Fig. 4B-C). We did not find an effect of hypoxia on fibroblast contamination. Together, these results show that in low passage cultures, fibroblast contamination is high, but more importantly, in high passage cultures, fibroblast contamination is very low, and cultures mainly consist of satellite cells.

Gene expression of early satellite cell markers seems to be promoted by hypoxia

In qualitative gene expression analysis, we found that myogenic regulator factors *MYF5* and *MYOD1* were upregulated in cells cultured under hypoxia, while desmin (*DES*) and α-sarcomeric actin (*ACTA1*), a marker indicative for structural myogenic differentiation, were downregulated in cells cultured under hypoxia. For satellite cell markers *PAX7* and *NCAM* there was no difference detected (Fig. 5A). With quantative gene expression analysis, expression of *MYOD1* and *MYF5* was respectively

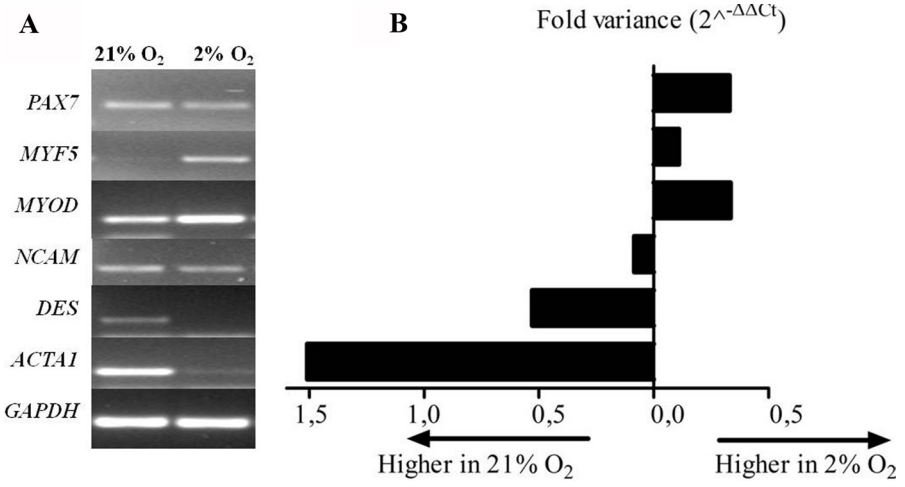


Figure 5. Gene expression analyses. (A) Qualitative gene expression analyses showed the differential regulation of myogenic genes by proliferating satellite cells during passage 3-15. (B) This was corroborated by quantitative analyses which showed that *PAX7*, *MYF5* and *MYOD1* were upregulated in cells cultured under hypoxia. *NCAM* was downregulated under hypoxia, as were *DES* and *ACTA1*. (N = 4; data are represented as means).

0.3 and 0.1 fold upregulated in cells cultured under hypoxia compared to normoxia. The expression of *DES* and *ACTA1* was downregulated respectively 0.5 and 1.5 fold. In contrast, *PAX7* was upregultated 0.3 fold, while *NCAM* was downregulated 0.1 fold in satellite cells that were cultured under hypoxia (Fig. 5B). The differences at gene transcriptional level between cells cultured under normoxia or hypoxia are not significant, however, figure 4 represents a trend that under hypoxic culture conditions, quiescent satellite cells are promoted.

Myogenic differentiation of satellite cells unaffected by hypoxia

To determine whether satellite cells can form myotubes under hypoxic culture conditions, we exchanged the proliferation medium to differentiation medium when cells had reached 100 % confluence in either oxygen concentrations.

We observed that cells in both culture conditions had started to fuse and form multinucleated cells that resembled myotubes within three days after myogenic induction, while both at day 3 and day 5 mononucleated cells were still present (Fig. 6A, B, C). The multinucleated cells as well as the mononucleated cells expressed desmin (Fig. 6D, E, F). However, only the multinucleated cells expressed myosin (Fig. 6G, H), which confirmed their being myotubes. Furthermore, after five days of differentiation, in high passage cell cultures, cross-striations in the myotubes became visible after

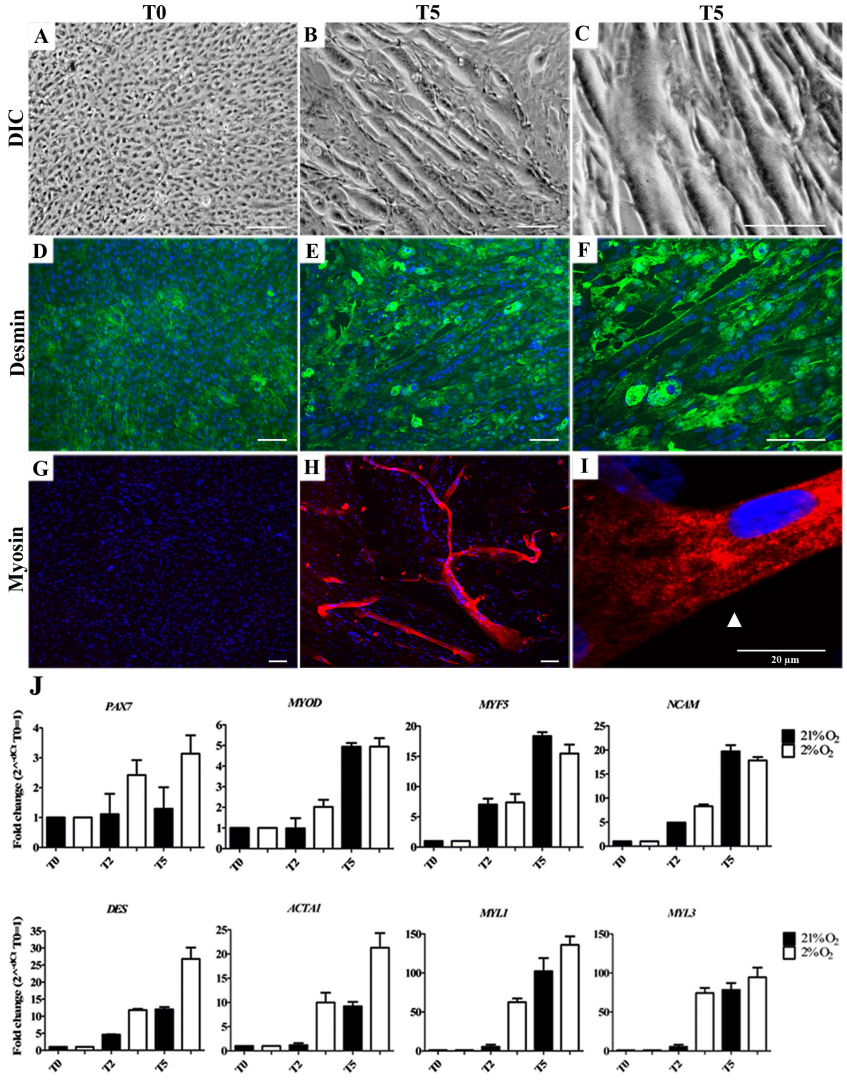


Figure 6. Differentiation of human satellite cells. (A, D, G) In proliferating satellite cells at high passage (P6-P15), differentiation was initiated when satellite cells reached 100 % confluence (T0) by swiching to differentiation medium. (B, C) Five days after switching to differetiation medium, myotubes were clearly visible by differential interference contrast (DIC) microscopy. (D, E, F) Desmin (green) was expressed by undifferentiated satellite cells as well as in myotubes, while (G, H) Myosin (red) was solely expressed in myotubes and not by mononucleated cells. (I) Cross-striations in myotubes were visible (arrowhead) which are reminiscent of striated muscle fibers. Nuclei are counterstained with DAPI (blue). (J) During differentiation of satellite cells into myoblasts and myotubes, qRT-PCR analysis showed a slight (2 to 20-fold) upregulation of *PAX7*, the myogenic regulator factors *MYF5* and *MYOD1*, and *NCAM*. Genes of myogenic differentiation *DES, ACTA1, MYL1* and *MYL3* show a strong (up to 150-fold) upregulation. During differentiation, gene regulation was comparable between both oxygen conditions, though all genes tended to be more upregulated under hypoxia. The protein expression pattern of Myosin concurs with the upregulated gene expression of *MYL1* and *MYL3* during myogenic differentiation. (N = 4; data are represented as means ± SEM) Scalebars are 100 µm.

staining for myosin (Fig. 6I). These are the hallmarks of striated muscle.
During differentiation, the morphology of cells was not affected by the oxygen concentration: in normoxic culture conditions there was differentiation of satellite cells and myotube formation, equally efficient in hypoxic culture conditions based on myotube size and increase in MyoD positive nuclei (data not shown).
Quantitative gene expression analysis showed an upregulation of *PAX7*, the myogenic regulator factors *MYF5* and *MYOD1*, and *NCAM* during differentiation. Genes of myogenic differentiation such as *DES, ACTA1, MYL1* and *MYL3* showed a twenty to hundred fold upregulation concomitant with myotube formation. During differentiation gene regulation is comparable between both oxygen conditions, though all genes are more upregulated by cells cultured under hypoxia (Fig. 6J).
The protein expression pattern of myosin matches the upregulation of *MYL1* and *MYL3*, genes responsible for myosin expression.

Myogenic differentiation predisposes a fraction of satellite cells to quiescence.

In differentiation medium, part of the satellite cells did not fuse into myotubes. To determine whether these cells remained activated satellite cells or whether they acquired a quiescent phenotype, an immunofluorescent staining for Pax7 was performed which was quantified by TissueFAXS analysis. Data showed that during myotube formation the percentage Pax7 positive cells did not change (Fig. 7A-B). However, the Mean Fluorescent Intensity of Pax7 positive cells had increased (Fig. 7B-C). This increase in expression of Pax7 occurred only in the remaining mononucleated cells, but not in the myotubes (Fig. 7D). This observation concurs with our findings that gene expression of *PAX7* is upregulated during myogenic differentiation.

DISCUSSION

The current study set out to investigate the influence of hypoxia on proliferation and differentiation of human satellite cells. One major finding is that hypoxia increases proliferation of low passage human satellite cells *in vitro*. Second, satellite cells cultured for multiple passages under hypoxia maintain their myogenic properties and enhance their functional capacity of differentiation towards myotubes. Finally, another important finding is that during myogenic differentiation *in vitro*, part of the satellite cells acquired a quiescent phenotype. Taken together, these findings render human

satellite cells highly suitable for tissue engineering of skeletal muscle for clinical applications.

The proliferation rate of satellite cells during passages 0-5 was higher under hypoxia than normoxia, which may imply that satellite cells overgrow fibroblasts quicker under hypoxia. At higher passage number, cultures consist of mainly satellite cells and proliferation rate increases 3 to 7 fold, irrespective of oxygen level. These observations *in vitro* correlate to the response of satellite cells *in vivo* where hypoxia induces vigorous proliferation too [145]. Other studies also reported a beneficial effect of low oxygen levels on the proliferation of satellite cells [158,160,161]. However, these investigators did not use human satellite cells, but a mouse cell line (C2C12), rat or bovine derived or whole muscle fibers. Moreover, in these studies only low passage satellite cells were used, and they were subjected to hypoxia after initial normoxic culture. In those circumstances we observed a similar, albeit, temporary increase in proliferation rate (Fig. 1A; Fig. 2). This is interesting in tissue engineering, because large numbers of cells are necessary and these cells must be able to withstand the hypoxic surroundings they will encounter to ensure survival. We have shown that satellite cells thrive on low levels of oxygen (2 % O_2).

A major finding of this study is that in cell populations derived of human skeletal muscle, Desmin^{pos}/NCAM^{pos} and Desmin^{pos}/NCAM^{neg} satellite cell populations are present. NCAM is an 'established' satellite cell marker which is frequently used to purify cell population by cell sorting [41,162]. However, in doing this, the Desmin^{pos}/NCAM^{neg} population is discarded, which are satellite cells capable of differentiation. Moreover, a heterogeneous population of satellite cells may have a distinct benefit, because their capacity to differentiate and form functional myotubes, *i.e.* generate force, requires supportive cells [43].

The finding that hypoxia decreases NCAM protein levels in satellite cells, was corroborated by gene transcript analyses. As it appeared, satellite cells cultured under hypoxia harbor a more quiescent state in terms of gene transcripts, while normoxia promotes a more activated myoblast gene expression profile during proliferation (Fig. 5). Consistent with our findings is the upregulation of *MYOD1* described in primary rat and bovine satellite cells cultured under hypoxia [158,163]. However, these published results contrast with the murine myoblastic cell line C2C12. Under hypoxia, C2C12 cells show a delayed myogenic differentiation due to a downregulation of *MYOD1*, which is one of the dominant myogenic transcription factors. This downregulation might be caused by an accelerated degradation of *MYOD1* at the post-transcription level by C2C12 [164]. However, this downregulation of *MYOD1* is transient, which

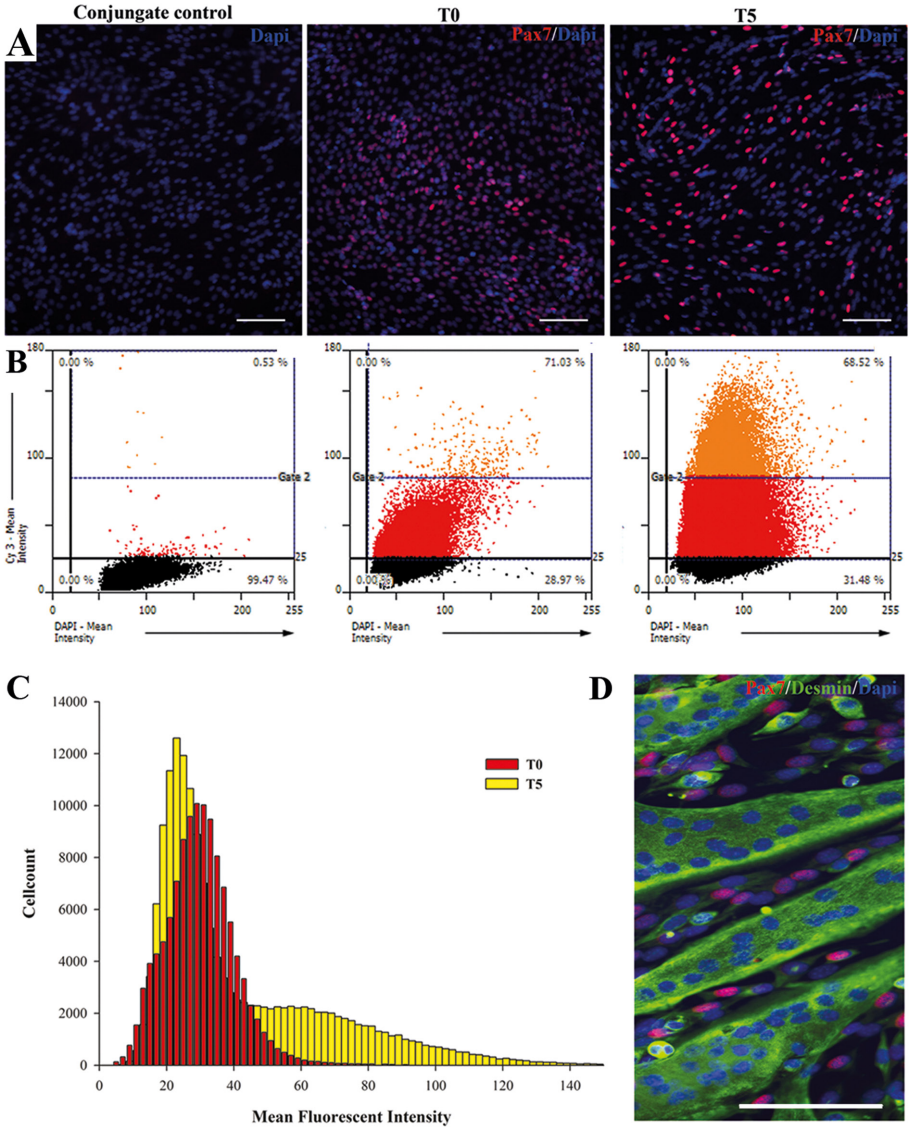


Figure 7. Pax7 expression is maintained during myogenic differentiation. (A-B) At day 0 (T0) myogenic differentiation was induced in confluent cultures of human satellite cells at high passage (P6-P15) and monitored on day 5 (T5). (B) Analyses by TissueFAXS showed that the fraction of Pax7 (red) expressing cells did not change during myotube formation. (B-C) However, the Mean Fluorescent Intensity increased in the remaining mononucleated cells at T5. (D) Remarkebly, Pax7 was not expressed by nuclei in Desmin (green) positive myotubes. This observation concurs with our observation that gene expression of *PAX7* was upregulated during myogenic differentiation. Nuclei are counterstained with DAPI (blue). Scalebars are 100 µm.

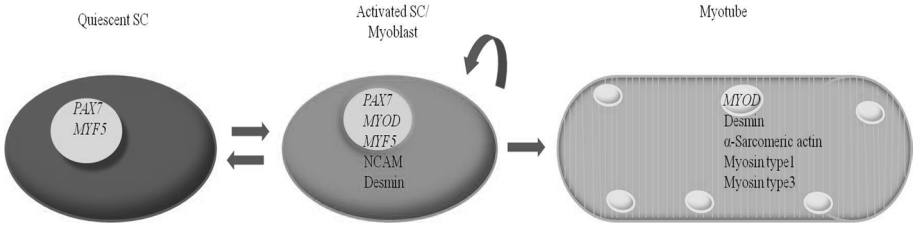


Figure 8. Transcriptional lineage of satellite cells. During differentiation of human satellite cells, myotubes are formed and gene and protein expression patterns shift towards mature muscle. However, some activated satellite cells differentiate into a quiescent state, with a cocurrent increase of Pax7 expression (Fig. 5 and 6). ***In vivo***, this would represent a mechanism for satellite cells to maintain a reserve of myogenic progenitor cells (quiescent satellite cells, or reserve cells). The oxygen concentration during culture of human satellite cells is not a significant regulating factor in differentiation. However, low oxygen concentration does promote *PAX7* expression, engaging satellite cells in myogenic lineage and promoting more efficient proliferation.

indicates that C2C12 myoblasts can adapt to hypoxic conditions too [163]. This indicates that C2C12 and human satellite cells respond differently to hypoxia. Furthermore, human satellite cells also show donor variation at protein and gene expression levels. The donor variation could be a reflection of the functional heterogeneity within subpopulations of satellite cells [135,136,141]. Also, due to their different embryonic origin, satellite cells derived from craniofacial muscle have properties that set them apart from satellite cells derived from limb muscles [48], which might also be a cause for our donor variation. Furthermore, there was some age variation in our research population. This is important, because when age increases, satellite cell population is decreased and their myogenic capacity reduced [131,133,134]. However, in this study, we could not relate the donor variation to muscle origin or donor age, because the variation was not significant and our population was too small.

During differentiation, mononucleated satellite cells formed multinucleated myotubes in both oxygen conditions. The myotubes expressed myosin, unlike mononucleated cells. Simultaneously, both *MYL1* and *MYL3*, genes responsible for respectively myosin type-1 and myosin type-2 expression were upregulated during differentiation. Both mono- and multinucleated cells expressed desmin, therefore, the remaining mononucleated satellite cells most likely retained the capacity to form myotubes. Alternatively, even under myogenic conditions desmin-expressing cells could (de) differentiate to quiescent satellite cells. This quiescent state was confirmed by the enhanced expression of Pax7 during myotube formation by mononucleated cells (Fig. 7). This also explains the upregulation of *PAX7* observed at gene transcript level. We can therefore conclude that mononucleated cells maintain their satellite cell character

or dedifferentiate towards their quiescent state (Fig. 8). We surmise that this *in vitro* synchronous bi-directional differentiation of satellite cells reiterates *in vivo* processes in which an appropriate niche of myogenic progenitor cells (quiescent satellite cells, or reserve cells [89]) is recreated upon regeneration of ischemic muscle tissue. Furthermore, this is in concordance with the findings derived from mice and rat tissue, that satellite cells can both self-renew and differentiate after transplantation [83,165]. Moreover, this process could be influenced by various signaling pathways promoting either myoblast and myotube formation or quiescent satellite cells [166-171]. This offers great potential for future clinical applications of tissue engineered muscle, where the presence of cells with a self renewing capacity is essential. Moreover, we find that these processes are not influenced by hypoxia, which satellite cells will encounter upon implantation.

For tissue engineering, where large cell numbers are required, hypoxia improves proliferation during low passage tremendously. We therefore recommend culturing human satellite cells under hypoxic conditions during the first passages.

CONCLUSION

Hypoxic culture conditions promote initial cell proliferation of human satellite cells. Moreover, while satellite cells differentiate into myotubes, they maintain a pool of quiescent satellite cells with qualities which are unaffected by hypoxia. Combined, this renders human satellite cells highly suitable for tissue engineering of skeletal muscle for future clinical application.

CHAPTER

6

IN VIVO MUSCLE MATURATION IN A HUMAN SATELLITE CELL ENGINEERED CONSTRUCT DOES NOT DEPENDENT ON PRECONDITIONING WITH HUMAN ENDOTHELIAL CELLS.

Merel Koning, Marja J.A. van Luyn, Daisy W.J. van der Schaft, Jacqueline Vulto, Paul M.N. Werker, Martin C. Harmsen

ABSTRACT

Innovative strategies such as tissue engineering of skeletal muscle constructs are promising for treatment of patients with skeletal muscle damage. How these human muscle constructs differentiate into mature muscle fibers *in vivo* remains to be elucidated. Therefore, we engineered 3D muscle constructs in collagen type I gels with clonally expanded human satellite cells. These constructs were preconditioned *in vitro* with 10 % human umbilical vein endothelial cells (HUVEC) for 5 days to investigate the effect on vascularisation *in vivo*.

Engineered constructs were implanted subcutaneously in nude rats for 1, 5, 10 or 21 days. After explantation, morphological analysis revealed progressive and extensive mature muscle fiber formation. Irrespective of the *in vitro* preconditioning with HUVEC, functional vascularisation was host-derived and equally efficient in both types of constructs. Analyses of myogenic regulator factors showed that muscle fiber formation and maturation occurred from the periphery of the construct inward. Transmission electron microscopy and qRT-PCR confirmed the formation of mature skeletal muscle during the 21 day time course.

In conclusion, this study shows advanced skeletal muscle fiber formation by human satellite cells *in vivo*. Most importantly, we show that the formation and engraftment of human skeletal muscle *in vivo* is independent of preconditioning with HUVEC *in vitro*. Tissue engineering of human skeletal muscle without the need of *in vitro* prevascularisation offers potential as a future treatment option for patients with longstanding facial paralysis.

INTRODUCTION

Tissue engineering of autologous skeletal muscle aims to develop an improved treatment for patients with muscular deficiencies such as in facial paralysis. In this condition paralyzed facial muscles impair facial expression and cause significant physical, psychological and social distress. Surgical reconstructive options are available but slim because there are no autologous donor muscles with the same characteristics as these small facial muscles, and genuine regeneration is not achieved [6,7]. Engineered skeletal muscle tissue on the other hand, may be customized and offers an opportunity for patients to obtain a more natural result, without causing donor site morbidity [44] or downsides of current free flap reconstructions such as undue swelling or even the autostatic syndrome [5].

Muscle tissue has an endogenous repair mechanism through myogenic progenitor cells, *i.e.* satellite cells. Upon stimulation such as damage, satellite cells proliferate and differentiate into myotubes and muscle fibers, to regenerate the damaged muscle [82-85,172]. These regenerative capacities of human satellite cells make them a suitable source for muscle tissue engineering [91,130].

However, for successful regeneration of functional muscle tissue that exceeds the thickness compatible with nutrition by diffusion, a vascular network is required to exchange oxygen and nutrients. If revascularisation of large muscle grafts, either transplanted or engineered, proceeds too slow for effective regeneration to occur, this leads to fibrosis and loss of tissue [173]. Even though satellite cells are resilient to hypoxic conditions [112], and facial muscles are relatively thin and small compared to other skeletal muscles, vasculature will be imperative for sustainability and growth of the muscle tissue.

Endothelial cells form vascular like structures *in vitro*, a process which is enhanced by co-culture with mesenchymal stem cells such as adipose derived stem cells or bone marrow derived stem cells [174,175], cardiomyocytes [176] or myoblasts [177,178]. Also when implanted subcutaneously, co-cultures of endothelial cells with mesenchymal stem cells or myoblasts improved blood perfusion and survival *in vivo* [179,180]. However, these studies mainly focus on endothelial cells and vascularisation, and in those that do implement satellite cells, the formation of skeletal muscle is underexposed.

A 3D muscle construct developed with collagen type I (ColI) gel offers an environment with extracellular matrix and may resemble the architecture of the cells natural niche *in vitro*. An advantage of this ColI construct is that it can be molded into shape,

cells can be seeded uniformly and it promotes cellular organization. Subsequent strain that cells experience during *in vitro* culture in this ColI construct, promoted the alignment of the murine cell line C2C12 and the endothelial cell line H5V [144]. Also cardiomyocytes that were cultured in this 3D ColI construct experience strain that promoted aligned architecture *in vitro* [181]. Although cell lines, cardiomyocytes and other animal derived cells shed light on general mechanisms such as differentiation and alignment, these cells do not exactly represent human satellite cells *in vitro* nor *in vivo* [147,182].

We engineered 3D skeletal muscle constructs *in vitro* with clonally expanded human satellite cells to investigate differentiation of satellite cells and myotubes, and subsequent muscle fiber formation *in vivo*. Furthermore, we investigated the effect of *in vitro* preconditioning of these muscle constructs with human umbilical vein endothelial cells (HUVEC) on muscle fiber formation and vascularisation *in vivo*.

MATERIALS AND METHODS

Satellite cell isolation and culture

A muscle biopsy was obtained from a 60 year old healthy female, undergoing blepharoplasty. The study protocol was approved by the institutional medical ethics committee, and the donor gave her informed consent. Satellite cells were isolated after enzymatically dissociation of the muscle biopsy. Dissociation was performed with 0.04 mg/ ml (0.16 Collagenase Wünsch units/ml) Liberase Blendzyme 3 (Roche Applied Science, the Netherlands). Satellite cells were proliferated with proliferation medium (PM) which consisted of Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen/ Gibco, CA, USA), 20 % Fetal Bovine Serum (FBS; Invitrogen/Gibco) and 1 % penicillin/streptomycin 50 µg/ml (Sigma-Aldrich, St. Louis, USA).

Differentiation medium (DM), contained DMEM, 2 % FBS, 1 % penicillin/streptomycin, 1 % Insulin-Transferrin-Selenium-A (100x) (Invitrogen) and 0.4 µg/ml dexamethason (Sigma-Aldrich). Medium was refreshed every other day. Cells were plated at 5.0 x 10^3 cells/cm^2 in culture flasks precoated with 1 % gelatine/PBS for 30 min. When cells reached 70 % confluence they were enzymatically harvested using Accutase (Invitrogen) and passaged.

Passage number (P_x) was defined as the xth sequential harvest of a subconfluent cell population. At passage 8, cells were cloned by sorting single cells in 96 wells plates using a MoFlow FACS. A clone that uniformly expressed the satellite cell marker

Pax7 and the myogenic regulator factors MyoD and Myogenin was selected for all experiments.
Human umbilical cord endothelial cells (HUVEC) were obtained through Prof. Dr. G. Molema (Dept. Pathology and Medical Biology, UMC Groningen, The Netherlands). HUVEC were cultured in gelatin-coated culture flasks in endothelial cell medium (ECM) until they reached confluence. ECM comprised of RPMI 1640 (Cambrex, NJ) supplemented with 20 % FBS, 2 mM L-glutamine (Sigma, St. Louis, MO), 1 % penicillin/streptomycin, 5 U/mL heparin (Leo Pharma, The Netherlands) and 5 mg/mL endothelial cell growth factors (ECGF; own isolate according to Burgess et al [183]). HUVEC cultures typically contained > 98 % cells expressing endothelial markers including VE-Cadherin and CD31, while sprouting networks formed within 6 hours on Matrigel® (BD Biosciences).

3D muscle construct engineering

Muscle constructs were cultured as previously described [105]. Briefly, house-shaped pieces of Velcro were glued to the bottom of a 6 wells plate 12 mm apart, sterilized with ethanol (70 %) and subsequent exposed to UV for 15 min. A gel mixture was prepared on ice by mixing 50 % Collagen type I (3,44 mg/ml, BD Biosciences), 39 % PM, 3 % 0.5 M NaOH (Sigma-Aldrich), 8 % growth factor reduced Matrigel. Three groups were cultured: a bare construct without cells, a construct with satellite cells, a construct with a mixture of satellite cells and HUVEC. Cells were harvested and mixed with the gel at a concentration of 4.5×10^6 per ml satellite cells and 4.5×10^5 per ml HUVEC. Then, 350 µl of gel mixture was pipetted in and between the Velcro anchoring points. After 1 hour gelation, 3 ml PM was added which was replaced after 24 hours with DM, and refreshed daily for 5 days at which point the implantation in rats was performed.

Animal experiments

Male, athymic nude rats (Harlan, the Netherlands), 6 weeks of age, were held under specific pathogen-free conditions in individual ventilated cages and received food and water ad libitum. Animal experimentation was approved by the local Ethical Committee on Animal Experiments (DEC5729) and was conducted according to governmental and institutional guidelines on animal experimentation.
Rats were anesthetized with a mixture of isofluorane (Forene/Abbott BV, the Netherlands) and oxygen, and received 0,01 mg/kg Temgesic sc. per-operative. The back was disinfected and subcutaneous pockets were made to the left or right flank through

5 mm incisions. The constructs were implanted 1cm from the site of incision. Incisions were closed with a 5.0 Viclyl suture (Ethicon, VWR International). At day 1, 5, 10 or 21 after implantation, rats were anesthetized and the muscle constructs were explanted along with sample surrounding tissue (N = 6 per time point). The explants were fixed in either 2 % glutaraldehyde (in 0.1 M sodium phosphate buffer), 10 % formaldehyde or liquid nitrogen for further tissue processing.

Immunofluorescent staining

For histochemical analyses, glutaraldehyde-fixed explants were dehydrated, resin (Technovit 7100, Heraeus Kulzer, Germay) embedded, sectioned at 2 μm and stained using toluidine blue according to standard procedures. Toluidine blue-stained sections were randomized and evaluated for cellular density.

For immunohistochemical analysis, sarcomere components were detected with mouse-anti-human α-sarcomeric actin (1:100) (Sigma-Aldrich), mouse-anti-human myosin (MF20; 1:500) (Developmental Studies Hybridoma Bank, Iowa, USA (DSHB)) and rabbit-anti-human Desmin (1:100) (Novus Biological, Littleton, USA) in 4 μm sections which were prepared from paraffin-embedded explants, deparaffinized before staining.

Myogenic transcription factors were detected with mouse-anti-human MyoD (1:100) (Dako, Glostrup, Denmark), mouse-anti-human Myogenin (F5D; 1:500) (DSHB) and mouse-anti-human Pax7 (1:10) (DSHB). And endothelium was detected with mouse-anti-rat His52, and mouse-anti-human CD31 (1:100) (Dako) in 4 μm sections prepared from frozen explants. After three washes with 0.05 % Tween in PBS, sections were incubated with a secondary antibody-cocktail constituted of FITC-conjugated goat-anti-rabbit IgG (1:100) (Southern Biotech, AL, USA), and Alexa Fluor®555 goat-anti-mouse IgG1 or IgG2b (1:300) (all Invitrogen) and 10 % normal human serum in PBS/DAPI. Sections were mounted in Citifluor AP1 (Agar Scientific, Essex, UK). Three images of each type of construct of day 5, 10 and 21 were analyzed for the orientation of the cells in the constructs with an in-house algorithm as previously described [144]. In short, for each pixel, the probability that it belongs to a structure was calculated, followed by an estimation of structure orientation. The algorithm was implemented in Mathematica (Wolfram). The orientation was plotted in a histogram.

Gene transcript analysis

Total RNA was isolated from frozen samples using the RNeasy Kit (Qiagen Inc., CA, USA), in accordance to the manufacturers protocol. Briefly, cells were lysed and dilu-

ted with an equal volume of ethanol (70 %). RNA was collected on an RNA binding filter by centrifugation. DNA was removed by incubation with a DNase I solution at 37 °C for 15 min. The RNA-binding filter was washed twice and subsequently the RNA was eluted with 14 µl Elution Buffer. The RNA concentration and purity were determined by spectrophotometry (NanoDrop Technologies, Wilmington, NC). For qRT-PCR analysis, total RNA was reverse transcribed using the First Strand cDNA synthesis kit (Fermentas UAB, Lithuania). In summary, 1 µg of total RNA was diluted in a final reaction volume of 20 µl containing random hexamer primer (0.5 µg), RiboLock™ Ribonuclease Inhibitor (20 U), 1 mM dNTP mix, and incubated at 37 °C for 1 h. The reverse transcription reaction was terminated by heating the mixture to 70 °C for 10 min, after which the samples were placed on ice. Quantitative RT-PCR analysis was performed in a final reaction volume of 10 µl, consisting of SYBR Green Supermix (Bio-Rad, Hercules, USA), 0.5 mM primer-mix (Table 1) and 5 ng cDNA. Reactions were performed at 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s, for 40 cycles. All human sequences were confirmed unique and non crossreactive by BLASTn analysis. Data were analyzed using Science Detection Software 2.2.2. To determine differences in expression, Ct-values were normalized against GAPDH-expression using the ΔCt-method ($\Delta Ct_{(gene)} = Ct_{(gene)} - Ct_{(GAPDH)}$). Fold change was calculated against the ΔCt of SC explants at day 1. All cDNA samples were amplified in triplicate, and 3 separate constructs per construct type were used.

Table 1. Primer sequence quantitative reverse transcription polymerase chain reaction.

Primer	Forward	Reverse
PAX7	ATCCGGCCCTGTGTCATCTC	CACGCGGCTAATCGAACTCA
MYOD1	AGCACTACAGCGGCGACTCC	CACGATGCTGGACAGGCAGT
DES	CATCGCGGCTAAGAACATTT	TGTTGTCCTGGTAGCCACTG
ACTA1	GCCGCGATCTCACCGACTA	GCTGTTGTAGGTGGTCTCGTGAA
MYL1	AAGCCCGCAATGCAGAAGAG	TTGCTTGCAGTTTGTCCACCA
MYL3	GAACACCAAGCGTGTCATCCA	TCAGCAGATGCCAGTTTTCCA
GAPDH	CTGCCGTCTAGAAAAACCTG	GTCCAGGGGTCTTACTCCTT

Statistics

All data are represented as mean ± SEM and statistical comparison between two groups was performed using the Student's t test. When comparing more than two groups one way ANOVA was used followed by the post hoc Bonferroni's multiple comparison test in Graph-Pad Prism Version 5 (GraphPad Software, Inc., La Jolla, CA, USA). Results were considered statistically significantly different for $p < 0.05$.

RESULTS

In vitro tissue engineering of a three dimensional muscle construct

Human satellite cells were isolated and cultured from enzymatically dissociated muscle tissue. Initial passages comprised of heterogeneous cell populations. To obtain a pure satellite cell population for *in vivo* experiments, cells were cloned and individual clones expanded; the fraction of human satellite cells that was able to clonally expand was 26.3 ± 4.0 %. A clone that uniformly expressed the satellite cell marker Pax7 and the myogenic regulator factors MyoD and Myogenin was selected for all experiments. Satellite cells differentiated *in vitro* in a collagen type I (ColI) gel and a three dimensional construct developed within 5 days (Fig. 1A). In both types of constructs, with or without HUVEC (10 satellite cells : 1 HUVEC), myotube formation was observed. The myotubes aligned parallel to the constraining direction (Fig. 1A-B, arrowheads). Double staining for desmin and myogenin showed a homogenic distribution of satellite cells and myotubes throughout the gel. The nuclei of these myotubes were still centrally located. The myogenin expression shows the pre-disposition of myotubes and mononuclear cells for muscle formation (Fig. 1B).

In vivo development of a muscle construct

The *in vitro* engineered muscle constructs, developed by mixing ColI with either human satellite cells alone or satellite cells and HUVEC, were evaluated after sub-

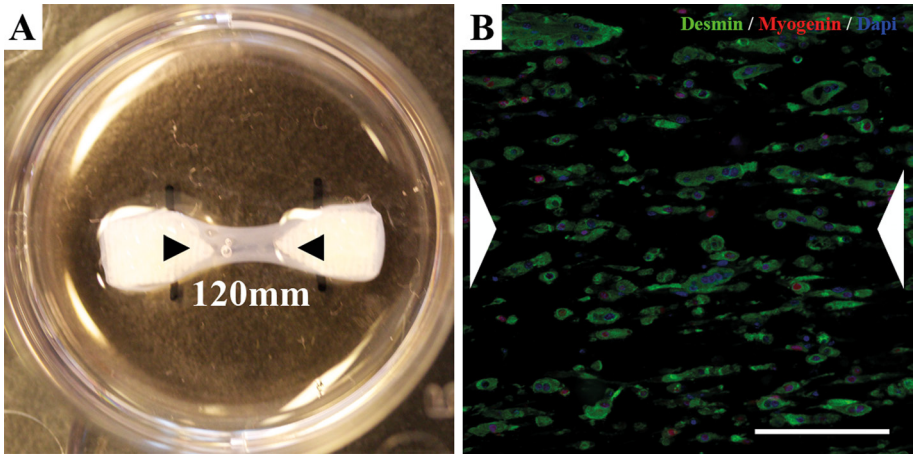


Figure 1. A tissue engineered muscle construct *in vitro*. (A) After 5 days of culture, the muscle construct consists of a compact gel between two fixed velcro points (arrowheads) approximately 120 mm apart. (B) An immunofluorescent image of a muscle construct shows that satellite cells and myotubes express Desmin (green) and Myogenin (red). Nuclei are counterstained with DAPI (blue). Scalebar is 200 µm.

cutaneous implantation in athymic nude rats. At all timepoints HUVEC were present in the explants of the HUVEC loaded muscle constructs (Fig. 2). After 1 day, the cell membrane and nucleus of most satellite cells within the implanted construct were sharply defined, indicating viability (Fig. 4A-B). Cells displayed the same healthy morphology in both types of constructs. After 5 days, myotubes had clearly widened, elongated and clustered (Fig. 4C-D). This process continued and at day 10, the number, diameter and length of myotubes had further increased in both types of constructs. Though myotubes were located predominantly at the outer layer of the constructs, at this time point they also started to appear in the center. In the bare implanted ColI gels, cellular infiltration was sporadically observed (Fig. 3), while in the cell-loaded gels, the ratio between cells and ColI gel had shifted and mostly cells and myotubes are seen (Fig. 4E-F). The development of muscle tissue continued and at day 21 the myotubes present throughout the constructs were striated and had flattened nuclei close to the sarcolemma (Fig. 4G-H).

Functional vascularisation of muscle constructs *in vivo*

We investigated the influence of preconditioning with or without HUVEC *in vitro* on the vascularisation of muscle constructs *in vivo*. At macroscopic level, the muscle constructs had compacted from 120 mm long by 0.5 mm thin structures to round shaped structures approximately 5 mm in diameter. Vascularisation of the constructs was comparable to the surrounding tissue. At microscopic level, the outer layers of both types of constructs were vascularised after 5 days (Fig. 4D.1), while in bare constructs vascularisation was virtually absent (Fig. 3). At day 10 and 21 vascularisation had further developed with increased numbers of vessels present throughout the construct and also in-growth of large vessels (Fig. 4E). These neovessels originated from the

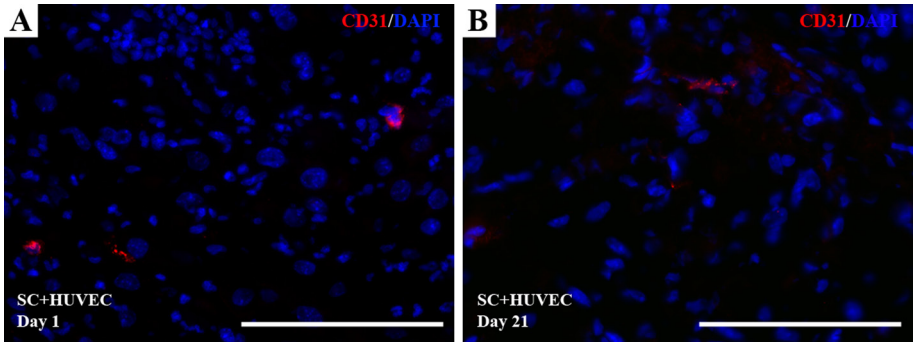


Figure 2. Immunofluorescent staining for CD31 (red) of explanted collagen I muscle constructs preconditioned with HUVEC at (A) day 1 and (B) day 21 after implantation. Nuclei were counterstained with DAPI (blue). Scalebars are 100 µm.

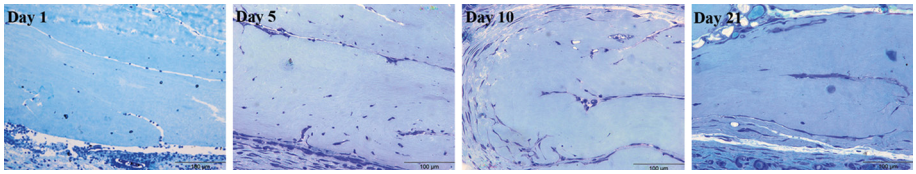
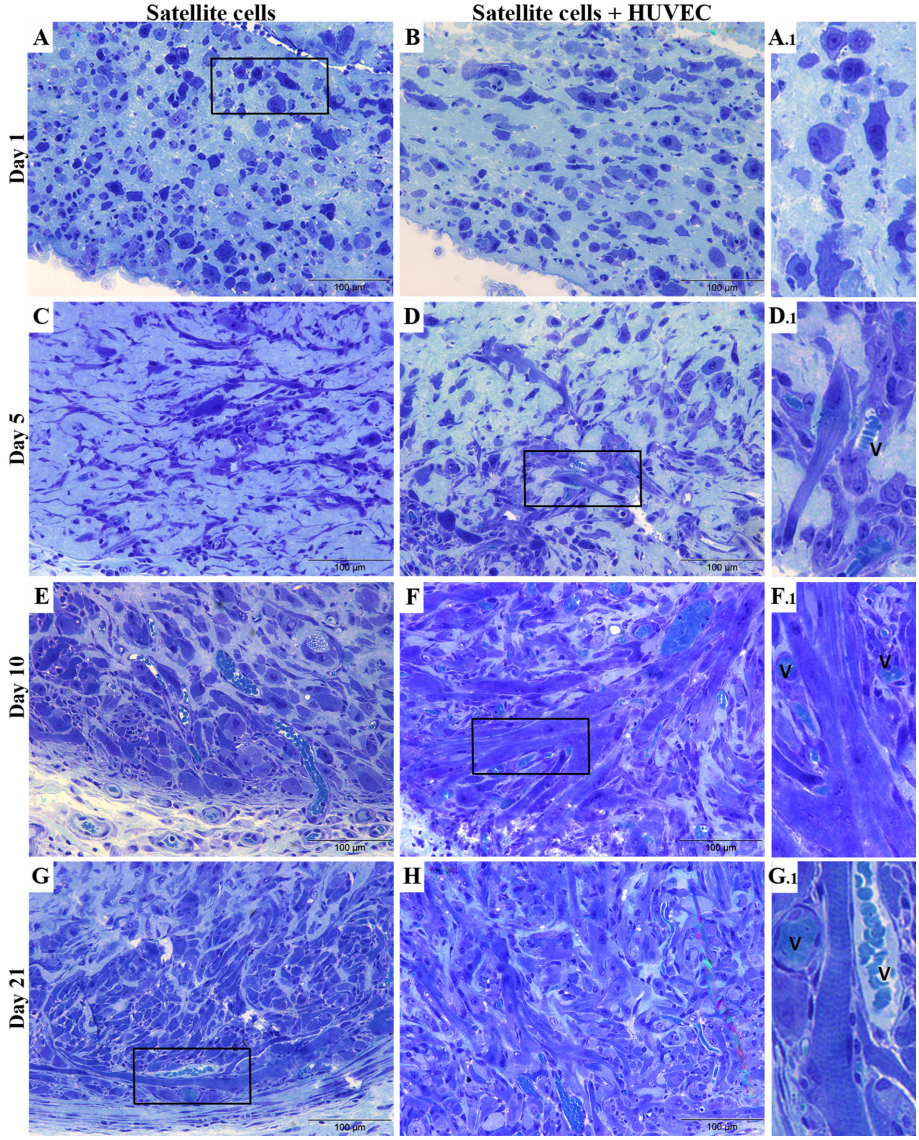


Figure 3. Toluidine blue staining of cross sections of explanted bare collagen I constructs. Note that there is little infiltration with host cells, virtually no ingrowth of host vasculature, and hardly any change between the different timepoints.



host vasculature, and were functional as indicated by the presence of erythrocytes in the lumen. Furthermore, some of the large vessels aligned with the formed muscle fibers (Fig. 4G.1).

Immunostaining for rat endothelial cells showed ingrowth of rat derived vasculature in the muscle construct at day 5 (Fig. 5A). This host-derived vascularisation occurred in constructs with and without HUVEC equally (Fig. 5B, C). At day 21, host-derived vessels had fully aligned with the human muscle fibers (Fig. 5D). Transmission electron microscopic (TEM) analysis also showed the integration of functional vessels in between muscle fibers (Fig. 5E). To investigate the stimulus for angiogenesis we analyzed quantitative gene expression of *VEGF* that was produced in both types of muscle constructs. This showed that at day 1 *VEGF* production was 8.4 ± 4.5 fold higher in constructs with HUVEC. However, the differences were not significant (Fig. 5F).

Skeletal muscle fiber formation by human satellite cells *in vivo*

TEM was employed to examine the ultrastructure of the muscle fibers that had formed in the constructs. We observed that myotubes formed mature muscle fibers that consisted of several tightly aligned myofibrils (Fig. 6A). The aligned myofibrils resulted in a cross-banded appearance which is typical for adult skeletal muscle. The dark A-bands were of constant length and contained a less dense central zone, the H-band, which was marked in the middle by the M-line. The I-band (which is known to consist of actin filaments) was bisected by a dense Z-line, where the filaments were joint laterally. Mitochondria were arranged in longitudinal direction between myofibrils, or still scattered throughout less mature muscle fibers. Nuclei had an open chromatin structure and the cytoplasm was well developed, which reflects a high activity in these cells. The myotube fusion process into muscle fibers was confirmed by TEM analysis too (Fig. 6B).

During *in vivo* development of the muscle construct, we found that myotubes werelocated predominantly at the outer layer of the construct. The surface of this outer layer increased compared to the total surface of a construct when measured in a cross section.

Figure 4. Development of a muscle construct after subcutaneous implantation. Toluidine blue staining of cross sections of muscle constructs (A, C, E, G) with human satellite cells alone or (B, D, F, H) with satellite cells and HUVEC. At day 1 (A, B), satellite cells and myotubes have round morphology (A.1). At day 5 (C, D), myotubes elongate, and in both types of constructs, with or without HUVEC, small vessels (**V**) appear in the outer layers (D.1). At day 10 (E, F), elongated thick muscle fibers form, and large vessels invade both types of constructs (F.1). At day 21 (G, H), muscle fibers display cross-striations, and vessels are present throughout the whole construct. (G.1) Erythrocytes are seen in the vessels. Scalebars are 100 µm.

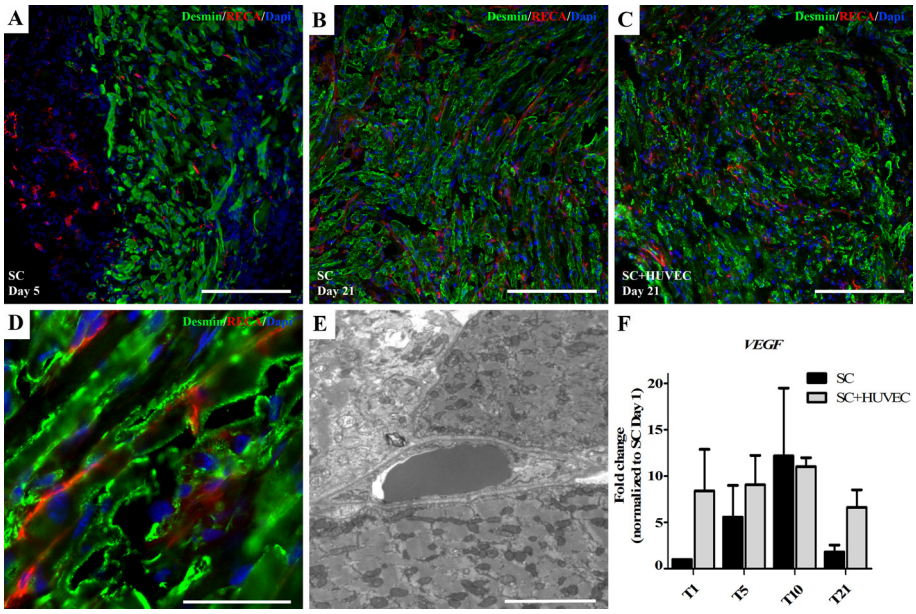


Figure 5. Functional vascularisation of muscle constructs *in vivo*. (A-D) Immunofluorescent staining for rat endothelial cells (RECA; red) and human muscle cells (desmin; green), nuclei were counterstained with DAPI (blue). (A) Day 5 showed ingrowth of rat derived vasculature in the muscle construct. (B, C) Day 21 showed rat derived vasculature throughout both types of muscle constructs. (D) Detail of a vessel wrapped around a muscle fiber. (E) TEM shows a vessel located between three adjacent muscle fibers in cross section. Erythrocytes are present in the lumen. Scalebars are 200 µm, 50 µm and 10 µm respectively. (F) *VEGF* production at gene expression level. (N = 3; data are represented as means ± SEM).

After 5 days, 5.2 ± 3.7 % of the total surface of satellite cell only constructs consisted of a layer of myotubes. After 21 days, this layer increased to 51.3 ± 1.5 % of the total cross sectional surface area. In constructs with HUVEC added, after 5 days, this layer was 18.1 ± 1.8 %, while after 21 days, this layer increased to 33.9 ± 1.6 % of the total surface. This increase however, was less than in satellite cell only constructs ($p = < 0.01$; Fig. 6C).

Myogenic regulator factors in human satellite cells promote differentiation *in vivo*

At all timepoints the satellite cell marker Pax7 was present throughout both types of muscle constructs (Fig. 7A-B). In general, both myogenic regulator factors myogenin and MyoD were predominantly expressed by satellite cells and myotubes located in the outer layer and at places where curvature of the construct was most pronounced. At day 21, myotubes that expressed myogenin were present throughout the construct. This was comparable for both types of constructs. Myotubes that had fused into mature muscle fibers, showed a decreased expression of myogenin, as well as flat-

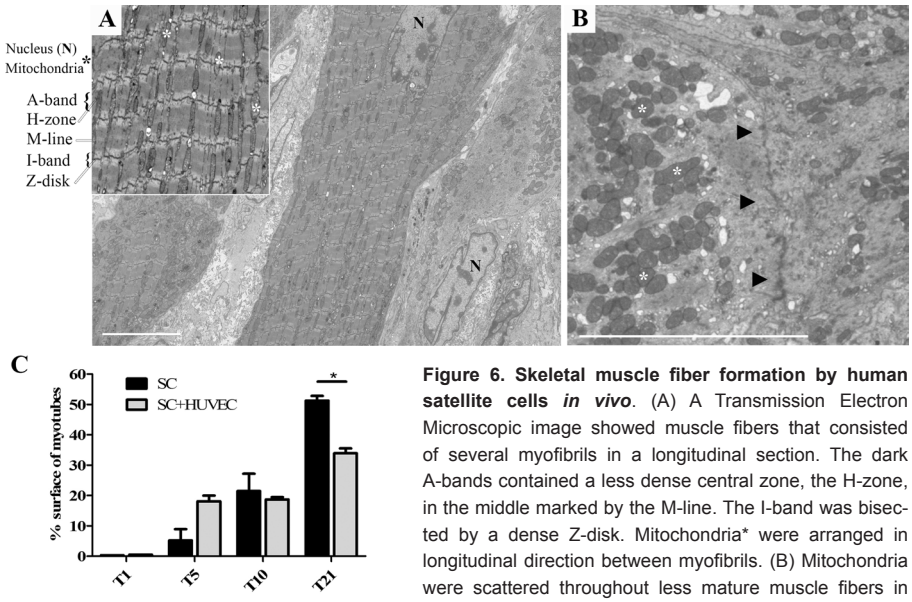


Figure 6. Skeletal muscle fiber formation by human satellite cells *in vivo*. (A) A Transmission Electron Microscopic image showed muscle fibers that consisted of several myofibrils in a longitudinal section. The dark A-bands contained a less dense central zone, the H-zone, in the middle marked by the M-line. The I-band was bisected by a dense Z-disk. Mitochondria* were arranged in longitudinal direction between myofibrils. (B) Mitochondria were scattered throughout less mature muscle fibers in fusion with one another (arrowheads). Scalebars are 10 µm. (C) The surface of myotubes and muscle fibers was compared to the total surface of a construct measured in a cross section (N = 3; data are represented as means ± SEM; * p = < 0.01).

tened nuclei on the outer layer of the fiber (Fig. 7C). Maturation of muscle fibers was most pronounced in the outer layer of the muscle constructs. At day 21, satellite cells and myotubes that expressed MyoD were present throughout both types of muscle constructs (Fig. 7D).

The increased expression of myogenic regulator factors by satellite cells and myotubes, was followed by mature muscle fiber formation and a subsequent increase in the expression of maturation markers such as desmin, α-sarcomeric actin and myosin. Moreover, these myotubes had aligned, which increased during *in vivo* maturation at the edge of the constructs (Fig. 7E boxed areas; E.1). Both quiescent satellite cell maintenance as muscle fiber development and alignment, were not influenced by the preconditioning with HUVEC.

This was largely confirmed by quantitative gene expression analysis of explanted muscle constructs. The satellite cell marker *PAX7*, the myogenic regulator factor *MYOD1* and genes of myogenic differentiation *DES, ACTA1, MYL1* and *MYL3* were upregulated during maturation *in vivo*. For all genes but *MYL1*, this upregulation was similar in constructs with HUVEC (Fig. 8). Therefore, also at gene expression level, myogenic regulator factors and mature muscle markers showed muscle fiber formation.

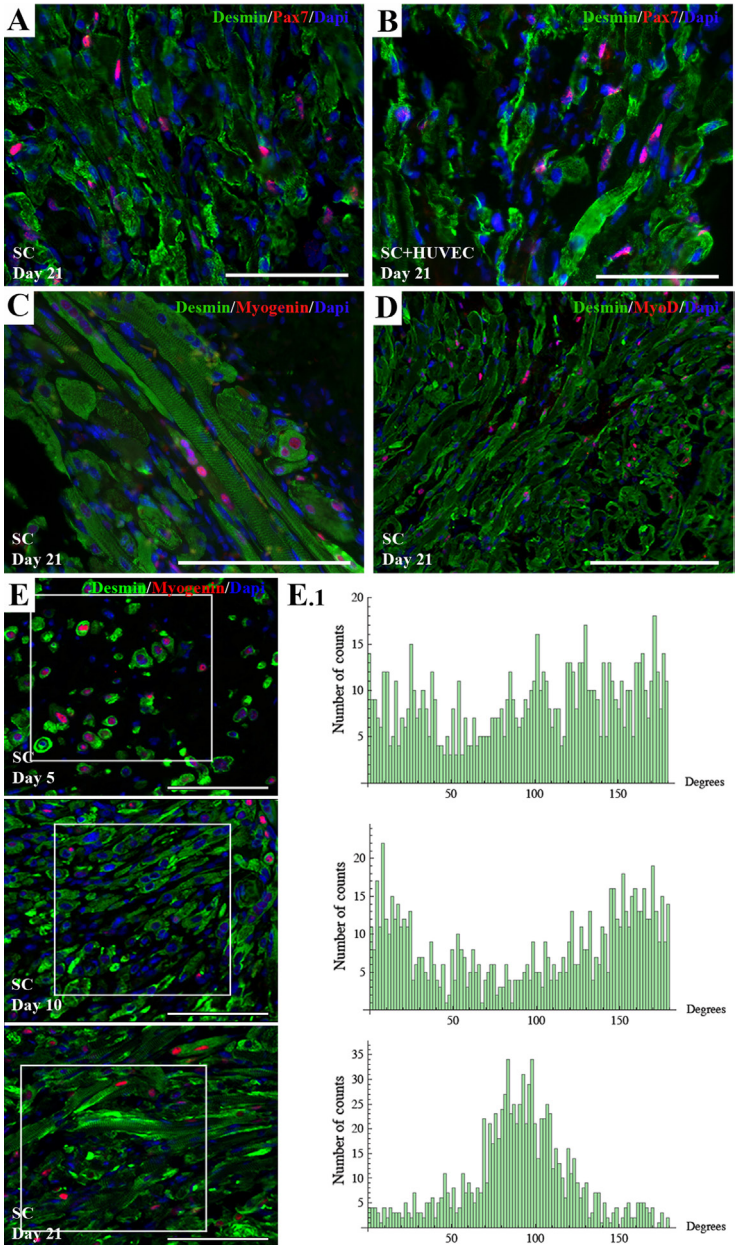


Figure 7. Myogenic regulator factors in human satellite cells promote differentiation *in vivo*. (A-B) Cross sections of muscle constructs with (B) or without (A) HUVEC, stained for Desmin (green) and Pax7 (red) at day 21. (C) Immunofluorescent staining of Desmin (green) and Myogenin (red) at day 21 showed terminally differentiated muscle fibers. (D) Immunofluorescent staining of Desmin (green) and MyoD (red) at day 21. (E) Immunofluorescent staining of Desmin (green) and Myogenin (red) at day 5, 10 and 21. The boxed area is quantified in E.1, where the number of pixels with a certain degree angle shows the alignment of myotubes and muscle fibers with each other. Nuclei were counterstained with DAPI (blue). Scalebars are 100 µm.

DISCUSSION

The current study set out to investigate the differentiation of clonally expanded human satellite cells and myotubes and subsequent muscle fiber formation subcutaneously in nude rats. *In vivo*, we showed that these satellite cells, after *in vitro* preconditioning in a 3D ColI gel, differentiate to form mature muscle fibers and quiescent satellite cells. A major finding was that the vascularisation of the mature human muscle fibers was host-derived vasculature. Furthermore, vascularisation immediately occurred upon implantation and proceeded along with maturation of the muscle constructs. Remarkably, *in vitro* preconditioning of the ColI muscle construct with HUVEC did not alter vascularisation, nor did it alter the differentiation of satellite cells or the muscle fiber formation *in vivo*.

Differentiation of satellite cells and myotubes into mature muscle fibers started at the outer layer of a muscle construct and then progressed inward (Fig. 4). Importantly, this muscle fiber formation was preceded by an upregulation in the myogenic regulator factors such as myogenin (Fig. 7C; E) and MyoD (Fig. 7D). At day 1, 5 and 10, the expression was predominantly located in the outer layers of the muscle construct, while at day 21 also centrally in the muscle construct cells expressed the myogenic regulator factors. The course of the muscle fiber formation could be explained in two ways. Firstly, the initiation and formation of myotubes during the *in vitro* preconditioning is likely promoted by the tension experienced by the satellite cells and myotubes in a muscle construct [144,181]. In addition, the expression pattern of myogenic regulator factors indicates that also *in vivo*, the increased tension that satellite cells and myotubes experience in the outer layers of the muscle construct, promotes muscle fiber formation. Interestingly, tension was also found to promote alignment of the murine cell line C2C12 [29]. We showed that also mature human muscle fibers align with each other (Fig. 6B).

Secondly, the *in vivo* formation of muscle fibers may be affected or depend on the coinciding vascularisation of the newly formed fibers [177]. This is supported by our observation that the outer layers are vascularised first by the host, they are therefore oxygenated earlier than the center of the construct. This contributes to muscle fiber formation [155]. Mononuclear satellite cells on the other hand are very resilient to hypoxic environments, they proliferate or become quiescent while maintaining their myogenic regenerative capacity (Fig. 7A) [112,158].

We choose to precondition muscle constructs with HUVEC *in vitro*, since endothelial cells contribute to neovascularisation through paracrine signaling [184]. Remarkably,

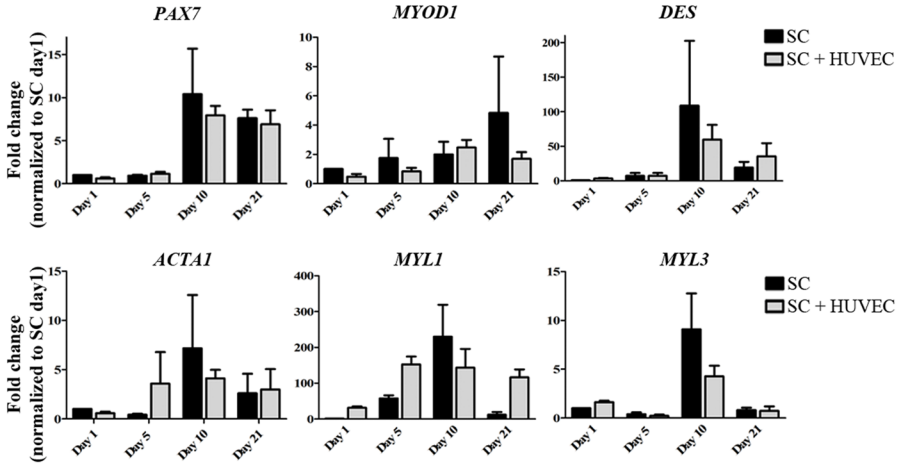


Figure 8. Quantitative gene expression analysis of explanted muscle constructs. (N = 3; data are represented as means ± SEM).

our results show that *in vitro* preconditioning of a muscle construct with HUVEC, did not improve vascularisation *in vivo*. Also muscle differentiation was not affected by the presence of HUVEC at 21 days. This suggests a higher self-reliance of human satellite cells compared to C2C12, as C2C12 do benefit from preconditioning *in vitro* for muscle fiber formation [144] and *in vivo* integration [25,179]. Interestingly, we found that only during the first 5 days after implantation preconditioning with HUVEC did ameliorate muscle fiber formation *in vivo* (Fig. 4C; D). After this, the difference in muscle fiber formation was even surpassed in the non-preconditioned muscle constructs. The initial head start might be a result of slightly increased VEGF production at day 1 by muscle constructs preconditioned with HUVEC (Fig. 5F). It is suggested that VEGF enhances myotube formation *in vitro* [185,186]. Also *in vivo*, it is suggested that VEGF enhances myotube formation [187,188] and engraftment of human myoblasts in mouse muscles [155]. However, during differentiation of satellite cells, VEGF production by satellite cells is upregulated [185]. Hence, our results indicate that VEGF from differentiating satellite cells regulates rat endothelial cell recruitment and vascularisation sufficiently [173,177,189-191]. Taken together, we show that *in vitro* preconditioning with HUVEC might be redundant in tissue engineering of small skeletal muscle tissue. The relatively thin and small facial muscles especially are a good model for the non-preconditioned muscle constructs. Larger skeletal muscles might still dependent on pre-vascularisation for their survival.

CONCLUSION

This study uniquely shows *in vivo* formation of mature muscle fibers and quiescent satellite cells by human satellite cells applied in an *in vitro* engineered muscle construct. Most importantly, we show that the formation and survival of mature human muscle is independent of preconditioning with HUVEC. Therefore, though innervation is a hurdle that remains to be addressed, this approach for tissue engineering of human skeletal muscle offers great potential treatment option for patients with long-standing facial paralysis.

CHAPTER 7

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

GENERAL DISCUSSION

Tissue engineering as a treatment option for longstanding facial paralysis

Significant loss of functional muscle tissue has a profound impact on a patient's life due to the limited capacity of self-repair of the muscle. Especially patients with longstanding facial paralysis suffer from a complex trilogy of physical, social and psychological symptoms. In a world where millions are spent on appearance, an asymmetric, paralyzed face causes severe psychological stress and even depression in some patients. It is estimated that every year approximately 4,000 people in the Netherlands are struck by facial paralysis. A large proportion of them recover spontaneously. Yet about 1,200 patients have persisting signs and symptoms. There are major advancements in the field of reconstructive surgery, and conservative, non-surgical treatment strategies [12]. However, only seldom can the asymmetry of the face be restored completely [10]. For these patients new, innovative treatment strategies need to be developed. The generation of skeletal muscle tissue using tissue engineering methods holds promise for the future treatment of these patients. Therefore, the main objective of this thesis was to improve tissue engineering of human skeletal muscle. We argued that new insights to promote and control tissue engineering of human skeletal muscle can be gained by studying human muscle progenitor cells, *i.e.* satellite cells, during their differentiation *in vitro* and *in vivo*.

Human satellite cells for tissue engineering of skeletal muscle

Skeletal muscle comprises of a mixture of both slow and fast twitching fibers, respectively myosin type I and type II. Facial muscles consist predominantly of fast type myosin [192,193].The differences between muscle fiber type also appear to be reflected in satellite cells. Their phenotype reflects the myosin type of the fiber of origin [194] (chapter 2). In this thesis we describe that human satellite cells clonally expand and have a relative fast proliferation rate *in vitro* (chapter 3, 5), thus theoretically, a single human satellite cell is sufficient to tissue engineer skeletal muscle for clinical application. Hence, only a small muscle biopsy is required to isolate satellite cells that are needed. We found that the part of the orbicularis oculi muscle that is removed during an upper eyelid blepharoplasty, is a sufficient source of satellite cells for tissue engineering purposes, and has virtually no donorsite morbidity.

This thesis shows fundamental regulatory processes of human satellite cells. We sho-

wed that, during *in vitro* differentiation of these cells, part of the population fuses and forms myotubes, while simultaneously another part returns to a quiescent state. Both processes are vital in tissue engineering: myotubes are required to form muscle fibers for contractility and force generation. And quiescent satellite cells are required to maintain their regenerative capacity, *i.e.* maintain the progenitor cell pool in engineered muscle.

Currently, two main theories that exist on the differentiation process of satellite cells: The first theory is that quiescent satellite cells undergo either symmetric cell division to maintain the progenitor cell pool or asymmetric cell division to contribute to muscle fiber repair [91]. This means that, when muscles are injured, quiescent satellite cells first proliferate and then either terminally differentiate into myotubes or dedifferentiate to replenish the quiescent satellite cell pool (Fig. 1A). The second theory is that upon damage, quiescent satellite cells are activated and start proliferating. These proliferating satellite cells then differentiate to terminal differentiated myotubes and quiescent satellite cells [146]. This means that, when muscles are injured, quiescent satellite cells go through an activated, proliferative stage. From this transitory proliferating population, most satellite cells undergo terminal differentiation while a few return to a stage to renew the quiescent satellite cell pool (Fig. 1B). Our findings, that a homogeneous clonally expanded cell population forms two different cell populations distinct from the original population (chapters 3 and 6), support the second theory.

MicroRNAs as a tool to tip the balance in favor of myotube formation

MicroRNAs have widely been accepted to play a role in tipping the balance in regulatory processes of various cell types. But as a tool to enhance (skeletal muscle) tissue engineering, microRNAs are a relative new concept. It was shown that by inhibition microRNA-133a in a murine myoblastic cell line C2C12, myotube formation was enhanced [143]. In chapter 3 of this thesis we showed that microRNAs are not involved in human quiescent satellite cell formation. However, in myotube formation both microRNA-1 and microRNA-206 are strongly upregulated. By further promoting these microRNAs in proliferating satellite cells even further (chapter 4), we tipped the balance and enhanced the potential for myotube formation. Also in a 3D muscle construct engineered from human satellite cells and collagen I gel, microRNA promotion enhanced myotube formation. This can become an important tool in tissue engineering. Especially with respect to aging, because with increasing age, the population of satellite cells per myofiber decreases [131,132]. Moreover, it has been shown that the myogenic capacity of satellite cells *in vitro* decreases during aging [132-134].

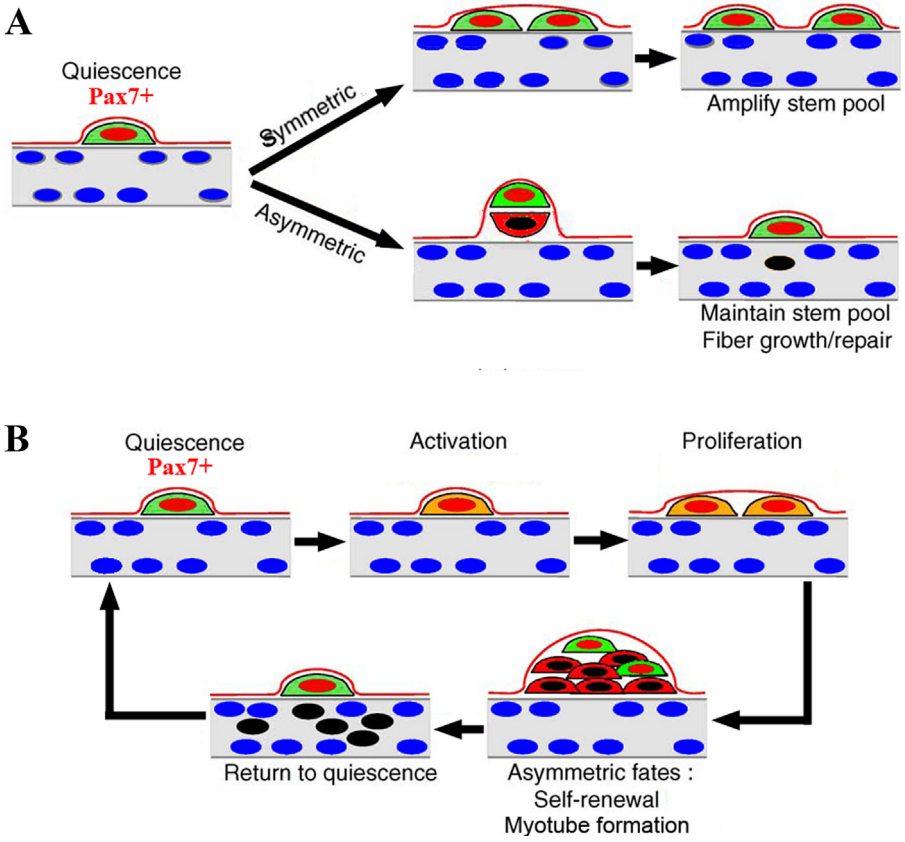


Figure 1. Two current models explaining the self-renewal and differentiation of satellite cells in muscles. (A) In a first model, Pax7^{pos} satellite cells co-exist with 'committed' satellite cells. Pax7^{pos} cells undergo symmetric cell division to amplify or maintain, respectively, the stem cell pool, while 'committed' satellite cells undergo asymmetric and terminal differentiation. (B) In a second model, quiescent (Pax7^{pos}) satellite cells go through an activated, proliferative stage. From this transitory proliferating population, most cells form myotubes and undergo terminal differentiation while a few return to a Pax7^{pos} stage to renew the quiescent satellite cell pool.

This implies that tissue engineering of skeletal muscle from autologous satellite cells from aged patients can be impaired. Furthermore, the efficiency of tissue engineering of skeletal muscle will vary between individual patients [135,136]. Overall, modulating satellite cell differentiation by promoting microRNAs might offer opportunities in enhancing the efficacy of successful tissue engineering of human skeletal muscle.

In vivo maturation of a tissue engineered human skeletal muscle construct

Upon clinical application, *i.e.* implantation of the engineered collagen I muscle

constructs, human satellite cells will encounter changes in oxygen level. How this will affect human satellite cell survival, proliferation and differentiation is largely unknown. In primary rat or bovine satellite cells hypoxic conditions promote myotube formation [158,163]. This is in contrast to C2C12 cells in which myotube formation is delayed due to a downregulation of *MYOD1* [164]. Chapter 5 reveals that hypoxic culture conditions enhanced proliferation of human satellite cells, without affecting their differentiation capacities. This is also an inspiring finding for *in vivo* applications of human satellite cells. Therefore, in chapter 6 we investigated differentiation and maturation of clonal human satellite cells in a 3D collagen I muscle construct implanted subcutaneously in nude rats. Subcutaneous implantation is a representative model for future application in patients with longstanding facial paralysis since in facial paralysis there is not a muscle defect but completely lacking muscle tissue.

It has been shown *in vitro* that in this type collagen I construct, C2C12 cells form myotubes that align parallel to the constraining direction [144]. We demonstrate subcutaneously in nude rats that clonal human satellite cells in collagen I constructs formed both aligned mature muscle fibers and quiescent satellite cells. Moreover, we showed that the muscle fibers were spontaneously and functionally vascularised by the host vasculature. This was in contrast to the bare collagen I gel, that showed no cellular infiltration or vessel formation, and served as an inert scaffold.

Levenberg et al. showed in nude mice that vascularisation of a C2C12 construct is promoted by human endothelial cells [25,179]. However, we found that host vascularisation of human muscle fibers was not promoted by the addition of human endothelial cells. Furthermore, Levenberg et al. also showed that vascula-

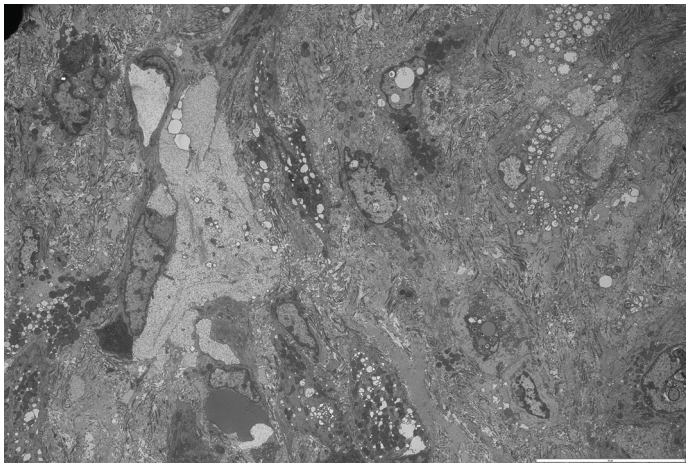


Figure 2. A Transmission Electron Microscopic image showed extracellular matrix formation in the human satellite cell muscle construct, and decreased muscle fiber formation after 21 days *in vivo*. Scalebar is 20 µm.

risation was promoted by additional support of embryonic human fibroblasts [25]. Yet in our hands (not shown in this thesis), the addition of supporting human adipose derived stem cells *in vivo*, only caused extracellular matrix formation in the muscle construct, and resulted in a decreased muscle fiber formation (Fig. 2). Taken together, the results of this thesis show that the murine myoblast cell line C2C12 greatly differs from primary human satellite cells. The translation from regulatory mechanisms in satellite cells to clinical application depends on the use and investigation of human satellite cells specifically in our view. Furthermore, we can conclude that clonal human satellite cells applied in a 3D collagen I gel is currently the optimal approach for tissue engineering of small human skeletal muscles. This offers potential for the treatment of small, thin facial muscles in patients with long-standing facial paralysis.

FUTURE PERSPECTIVES

In this thesis we showed that cloned human satellite cells in a collagen I construct form well vascularised mature muscle tissue after subcutaneous implantation in rats. However, various steps still have to be made to reach clinical application of such human muscle constructs.

To start, a more thorough analysis of human facial muscle characteristics could be performed to determine what prerequisites a tissue engineered muscle construct needs to meet. This means that the minimal contraction force that a construct should deliver and accurate size measurements of the different facial muscles have to be documented.

Through anatomical dissection and MRI investigation a general, but accurate picture of human facial muscles can be established. These studies will give a framework for the characteristics of facial muscles. Based on this framework, a combination of rapid prototyping, like 3D printing [195,196], and tissue engineering will hold promise for facial muscle repair in the individual patient.

Furthermore, though in our study human myotubes matured and formed muscle fibers *in vivo*, alignment of all the mature muscle fibers has to be observed to obtain a functional construct. If muscle fibers contract in a uniform direction, optimal force will be delivered. Alignment can be maintained if the human muscle construct remains strained during maturation *in vivo*.

Alignment is also related to integration of the human muscle construct with the inner-

vation. This will stimulate the human muscle construct, and promote alignment and contraction between muscle fibers. However, to reach functional innervation of a construct, two issues have to be addressed: first, the creation of a neuro-muscular junction, and second, the connection to the appropriate nuclei in the central nervous system. This will ultimately enable the patient to control movement of the integrated muscle construct. The first innervation issue can be studied during integration of differentiating satellite cells and neural cells in a transgenic rat expressing green fluorescent protein in peripheral nerves [197]. This model offers new possibilities to visualize the integration of innervation and a muscle construct during development in the host. The second innervation issue will be solved through cross-face nerve grafts. This way, the neural signal will be transferred from the healthy facial nerve to the affected side of the face and the newly tissue engineered muscle constructs.

Subcutaneous implantation in nude rats offers a good model for tissue engineering with the purpose of reanimating human facial muscles. However, eventually tissue engineered muscle constructs have to be tested in larger animal models. In a pig model, host derived vascularisation, alignment and innervation will be very representative for the human situation. Especially if facial paralysis is established first, this would be appropriate to study integration of a muscle construct, innervation and facial reanimation. Therefore, we can extrapolate the knowledge on well established rabbit models [198] in order to investigate facial reanimation after facial paralysis.

Although several steps have to be made before tissue engineering of autologous skeletal muscle will be clinically applicable, the fundamental knowledge and tools we have given in thesis hold promise and is worth further investing in.

CHAPTER 8

REFERENCES

[1] May M, Klein SR: Differential diagnosis of facial nerve palsy. Otolaryngol.Clin.North Am. 1991, 24: 613-645.
[2] Harrison DH: The pectoralis minor vascularized muscle graft for the treatment of unilateral facial palsy. Plast. Reconstr.Surg. 1985, 75: 206-216.
[3] Zuker RM, Goldberg CS, Manktelow RT: Facial animation in children with Mobius syndrome after segmental gracilis muscle transplant. Plast.Reconstr.Surg. 2000, 106: 1-8; discussion 9.
[4] Terzis JK, Karypidis D: Blink restoration in adult facial paralysis. Plast.Reconstr.Surg. 2010, 126: 126-139.
[5] Nicolai JP: Autostatic syndrome in association with facial nerve transplantation. Plast.Reconstr.Surg. 2003, 112: 1963-1964.
[6] Biglioli F, Frigerio A, Rabbiosi D, Brusati R: Single-stage facial reanimation in the surgical treatment of unilateral established facial paralysis. Plast.Reconstr.Surg. 2009, 124: 124-133.
[7] Terzis JK, Karypidis D: Outcomes of direct muscle neurotisation in adult facial paralysis. J.Plast.Reconstr. Aesthet.Surg. 2011, 64: 174-184.
[8] Mauro A: Satellite cell of skeletal muscle fibers. J.Biophys.Biochem.Cytol. 1961, 9: 493-495.
[9] Ambros V: microRNAs: tiny regulators with great potential. Cell 2001, 107: 823-826.
[10] Terzis JK, Noah ME: Analysis of 100 cases of free-muscle transplantation for facial paralysis. Plast.Reconstr.Surg. 1997, 99: 1905-1921.
[11] Kumar PA, Hassan KM: Cross-face nerve graft with free-muscle transfer for reanimation of the paralyzed face: a comparative study of the single-stage and two-stage procedures. Plast.Reconstr.Surg. 2002, 109: 451-462.
[12] Werker PM: [Plastic surgery in patients with facial palsy]. Ned.Tijdschr.Geneeskd. 2007, 151: 287-294.
[13] Vandenburgh HH: Functional assessment and tissue design of skeletal muscle. Ann.N.Y.Acad.Sci. 2002, 961: 201-202.
[14] Bach AD, Stem-Straeter J, Beier JP, Bannasch H, Stark GB: Engineering of muscle tissue. Clin.Plast.Surg. 2003, 30: 589-599.
[15] May M, Klein SR: Differential diagnosis of facial nerve palsy. Otolaryngol.Clin.North Am. 1991, 24: 613-645.
[16] Rubin LR: The anatomy of the nasolabial fold: the keystone of the smiling mechanism. Plast.Reconstr. Surg. 1999, 103: 687-691.
[17] Happak W, Liu J, Burggasser G, Flowers A, Gruber H, Freilinger G: Human facial muscles: dimensions, motor endplate distribution, and presence of muscle fibers with multiple motor endplates. Anat.Rec. 1997, 249: 276-284.
[18] Guyton AC, Hall J.E.: Textbook of Medical Physiology; 2000.
[19] Stal P: Characterization of human oro-facial and masticatory muscles with respect to fibre types, myosins and capillaries. Morphological, enzyme-histochemical, immuno-histochemical and biochemical investigations. Swed.Dent.J.Suppl. 1994, 98: 1-55.
[20] Dezawa M, Ishikawa H, Itokazu Y, Yoshihara T, Hoshino M, Takeda S, Ide C, Nabeshima Y: Bone marrow stromal cells generate muscle cells and repair muscle degeneration. Science 2005, 309: 314-317.
[21] Caplan AI: Review: mesenchymal stem cells: cell-based reconstructive therapy in orthopedics. Tissue Eng. 2005, 11: 1198-1211.
[22] DiEdwardo CA, Petrosko P, Acarturk TO, DiMilla PA, LaFramboise WA, Johnson PC: Muscle tissue engineering. Clin.Plast.Surg. 1999, 26: 647-64x.
[23] Dennis RG, Kosnik PE, Gilbert ME, Faulkner JA: Excitability and contractility of skeletal muscle engineered from primary cultures and cell lines. Am.J.Physiol Cell Physiol 2001, 280: C288-C295.
[24] Borschel GH, Dennis RG, Kuzon WM,Jr.: Contractile skeletal muscle tissue-engineered on an acellular scaffold. Plast.Reconstr.Surg. 2004, 113: 595-602.
[25] Levenberg S, Rouwkema J, Macdonald M, Garfein ES, Kohane DS, Darland DC, Marini R, van Blitterswijk CA, Mulligan RC, D'Amore PA, Langer R: Engineering vascularized skeletal muscle tissue. Nat.Biotechnol. 2005, 23: 879-884.
[26] Riboldi SA, Sampaolesi M, Neuenschwander P, Cossu G, Mantero S: Electrospun degradable polyesterurethane membranes: potential scaffolds for skeletal muscle tissue engineering. Biomaterials 2005, 26: 4606-4615.
[27] Huang NF, Patel S, Thakar RG, Wu J, Hsiao BS, Chu B, Lee RJ, Li S: Myotube assembly on nanofibrous and micropatterned polymers. Nano.Lett. 2006, 6: 537-542.
[28] Boontheekul T, Hill EE, Kong HJ, Mooney DJ: Regulating myoblast phenotype through controlled gel stiffness and degradation. Tissue Eng. 2007, 13: 1431-1442.

[29] Matsumoto T, Sasaki J, Alsberg E, Egusa H, Yatani H, Sohmura T: Three-dimensional cell and tissue patterning in a strained fibrin gel system. PLoS.ONE. 2007, 2: e1211.
[30] Dennis RG, Kosnik PE: Excitability and isometric contractile properties of mammalian skeletal muscle constructs engineered in vitro. In Vitro Cell.Dev.Biol.Anim. 2000, 36: 327-335.
[31] Beier JP, Kneser U, Stern-Strater J, Stark GB, Bach AD: Y chromosome detection of three-dimensional tissue-engineered skeletal muscle constructs in a syngeneic rat animal model. Cell Transplant. 2004, 13: 45-53.
[32] Stern-Straeter J, Bach AD, Stangenberg L, Foerster VT, Horch RE, Stark GB, Beier JP: Impact of electrical stimulation on three-dimensional myoblast cultures - a real-time RT-PCR study. J.Cell.Mol.Med. 2005, 9: 883-892.
[33] Das M, Gregory CA, Molnar P, Riedel LM, Wilson K, Hickman JJ: A defined system to allow skeletal muscle differentiation and subsequent integration with silicon microstructures. Biomaterials 2006, 27: 4374-4380.
[34] Borschel GH, Dow DE, Dennis RG, Brown DL: Tissue-engineered axially vascularized contractile skeletal muscle. Plast.Reconstr.Surg. 2006, 117: 2235-2242.
[35] Larkin LM, Van der Meulen JH, Dennis RG, Kennedy JB: Functional evaluation of nerve-skeletal muscle constructs engineered in vitro. In Vitro Cell.Dev.Biol.Anim. 2006, 42: 75-82.
[36] Huang YC, Dennis RG, Larkin L, Baar K: Rapid formation of functional muscle in vitro using fibrin gels. J.Appl.Physiol. 2005, 98: 706-713.
[37] Bach AD, Arkudas A, Tjiawi J, Polykandriotis E, Kneser U, Horch RE, Beier JP: A new approach to tissue engineering of vascularized skeletal muscle. J.Cell.Mol.Med. 2006, 10: 716-726.
[38] Kamelger FS, Marksteiner R, Margreiter E, Klima G, Wechselberger G, Hering S, Piza H: A comparative study of three different biomaterials in the engineering of skeletal muscle using a rat animal model. Biomaterials 2004, 25: 1649-1655.
[39] De CP, Delo D, Farrugia L, Udompanyanan K, Yoo JJ, Nomi M, Atala A, Soker S: Angiogenic gene-modified muscle cells for enhancement of tissue formation. Tissue Eng. 2005, 11: 1034-1044.
[40] Lewis MP, Tippett HL, Sinanan AC, Morgan MJ, Hunt NP: Gelatinase-B (matrix metalloproteinase-9; MMP-9) secretion is involved in the migratory phase of human and murine muscle cell cultures. J.Muscle Res.Cell. Motil. 2000, 21: 223-233.
[41] Sinanan AC, Hunt NP, Lewis MP: Human adult craniofacial muscle-derived cells: neural-cell adhesion-molecule (NCAM; CD56)-expressing cells appear to contain multipotential stem cells. Biotechnol.Appl.Biochem. 2004, 40: 25-34.
[42] Stern-Straeter J, Bran G, Riedel F, Sauter A, Hormann K, Goessler UR: Characterization of human myoblast cultures for tissue engineering. Int.J.Mol.Med. 2008, 21: 49-56.
[43] Brady MA, Lewis MP, Mudera V: Synergy between myogenic and non-myogenic cells in a 3D tissue-engineered craniofacial skeletal muscle construct. J.Tissue Eng Regen.Med. 2008, 2: 408-417.
[44] Shah R, Sinanan AC, Knowles JC, Hunt NP, Lewis MP: Craniofacial muscle engineering using a 3-dimensional phosphate glass fibre construct. Biomaterials 2005, 26: 1497-1505.
[45] Alessandri G, Pagano S, Bez A, Benetti A, Pozzi S, Iannolo G, Baronio M, Invernici G, Caruso A, Muneretto C, et al.: Isolation and culture of human muscle-derived stem cells able to differentiate into myogenic and neurogenic cell lineages. Lancet 2004, 364: 1872-1883.
[46] Jaspers RT, Feenstra HM, van Beek-Harmsen BJ, Huijing PA, van der Laarse WJ: Differential effects of muscle fibre length and insulin on muscle-specific mRNA content in isolated mature muscle fibres during long-term culture. Cell Tissue Res. 2006, 326: 795-808.
[47] Huang YC, Dennis RG, Baar K: Cultured slow vs. fast skeletal muscle cells differ in physiology and responsiveness to stimulation. Am.J.Physiol Cell Physiol 2006, 291: C11-C17.
[48] McLoon LK, Thorstenson KM, Solomon A, Lewis MP: Myogenic precursor cells in craniofacial muscles. Oral Dis. 2007, 13: 134-140.
[49] Illa I, Leon-Monzon M, Dalakas MC: Regenerating and denervated human muscle fibers and satellite cells express neural cell adhesion molecule recognized by monoclonal antibodies to natural killer cells. Ann.Neurol. 1992, 31: 46-52.
[50] Gussoni E, Soneoka Y, Strickland CD, Buzney EA, Khan MK, Flint AF, Kunkel LM, Mulligan RC: Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature 1999, 401: 390-394.
[51] Luth ES, Jun SJ, Wessen MK, Liadaki K, Gussoni E, Kunkel LM: Bone marrow side population cells are enriched for progenitors capable of myogenic differentiation. J.Cell.Sci. 2008, 121: 1426-1434.
[52] Asakura A, Seale P, Girgis-Gabardo A, Rudnicki MA: Myogenic specification of side population cells in

skeletal muscle. J.Cell Biol. 2002, 159: 123-134.
[53] Montarras D, Morgan J, Collins C, Relaix F, Zaffran S, Cumano A, Partridge T, Buckingham M: Direct isolation of satellite cells for skeletal muscle regeneration. Science 2005, 309: 2064-2067.
[54] Uezumi A, Ojima K, Fukada S, Ikemoto M, Masuda S, Miyagoe-Suzuki Y, Takeda S: Functional heterogeneity of side population cells in skeletal muscle. Biochem.Biophys.Res.Commun. 2006, 341: 864-873.
[55] Motohashi N, Uezumi A, Yada E, Fukada S, Fukushima K, Imaizumi K, Miyagoe-Suzuki Y, Takeda S: Muscle CD31(-) CD45(-) side population cells promote muscle regeneration by stimulating proliferation and migration of myoblasts. Am.J.Pathol. 2008, 173: 781-791.
[56] Saxena AK, Marler J, Benvenuto M, Willital GH, Vacanti JP: Skeletal muscle tissue engineering using isolated myoblasts on synthetic biodegradable polymers: preliminary studies. Tissue Eng. 1999, 5: 525-532.
[57] Sekine H, Shimizu T, Yang J, Kobayashi E, Okano T: Pulsatile myocardial tubes fabricated with cell sheet engineering. Circulation 2006, 114: I87-I93.
[58] Coletti D, Teodori L, Albertini MC, Rocchi M, Pristera A, Fini M, Molinaro M, Adamo S: Static magnetic fields enhance skeletal muscle differentiation in vitro by improving myoblast alignment. Cytometry A 2007, 71A: 846-856.
[59] Yan W, George S, Fotadar U, Tyhovych N, Kamer A, Yost MJ, Price RL, Haggart CR, Holmes JW, Terracio L: Tissue engineering of skeletal muscle. Tissue Eng. 2007, 13: 2781-2790.
[60] Kroehne V, Heschel I, Schugner F, Lasrich D, Bartsch JW, Jockusch H: Use of a novel collagen matrix with oriented pore structure for muscle cell differentiation in cell culture and in grafts. J.Cell.Mol.Med. 2008.
[61] Beier JP, Stern-Straeter J, Foerster VT, Kneser U, Stark GB, Bach AD: Tissue engineering of injectable muscle: three-dimensional myoblast-fibrin injection in the syngeneic rat animal model. Plast.Reconstr.Surg. 2006, 118: 1113-1121.
[62] Saxena AK, Willital GH, Vacanti JP: Vascularized three-dimensional skeletal muscle tissue-engineering. Biomed.Mater.Eng. 2001, 11: 275-281.
[63] Luttikhuizen DT, Harmsen MC, van Luyn MJ: Cellular and molecular dynamics in the foreign body reaction. Tissue Eng. 2006, 12: 1955-1970.
[64] Tsuda Y, Kikuchi A, Yamato M, Sakurai Y, Umezu M, Okano T: Control of cell adhesion and detachment using temperature and thermoresponsive copolymer grafted culture surfaces. J.Biomed.Mater.Res.A 2004, 69: 70-78.
[65] Nishida K, Yamato M, Hayashida Y, Watanabe K, Yamamoto K, Adachi E, Nagai S, Kikuchi A, Maeda N, Watanabe H, et al.: Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. N.Engl.J.Med. 2004, 351: 1187-1196.
[66] Yang J, Yamato M, Kohno C, Nishimoto A, Sekine H, Fukai F, Okano T: Cell sheet engineering: recreating tissues without biodegradable scaffolds. Biomaterials 2005, 26: 6415-6422.
[67] Yang J, Yamato M, Nishida K, Ohki T, Kanzaki M, Sekine H, Shimizu T, Okano T: Cell delivery in regenerative medicine: the cell sheet engineering approach. J.Control.Release 2006, 116: 193-203.
[68] Hatakeyama H, Kikuchi A, Yamato M, Okano T: Patterned biofunctional designs of thermoresponsive surfaces for spatiotemporally controlled cell adhesion, growth, and thermally induced detachment. Biomaterials 2007, 28: 3632-3643.
[69] Ohashi K, Yokoyama T, Yamato M, Kuge H, Kanehiro H, Tsutsumi M, Amanuma T, Iwata H, Yang J, Okano T, Nakajima Y: Engineering functional two- and three-dimensional liver systems in vivo using hepatic tissue sheets. Nat.Med. 2007, 13: 880-885.
[70] Masuda S, Shimizu T, Yamato M, Okano T: Cell sheet engineering for heart tissue repair. Adv.Drug Deliv. Rev. 2008, 60: 277-285.
[71] Neville CM, Schmidt M, Schmidt J: Response of myogenic determination factors to cessation and resumption of electrical activity in skeletal muscle: a possible role for myogenin in denervation supersensitivity. Cell. Mol.Neurobiol. 1992, 12: 511-527.
[72] Wagner S, Dorchies OM, Stoeckel H, Warter JM, Poindron P, Takeda K: Functional maturation of nicotinic acetylcholine receptors as an indicator of murine muscular differentiation in a new nerve-muscle co-culture system. Pflugers Arch. 2003, 447: 14-22.
[73] Dhawan V, Lytle IF, Dow DE, Huang YC, Brown DL: Neurotization improves contractile forces of tissue-engineered skeletal muscle. Tissue Eng. 2007, 13: 2813-2821.
[74] Powell CA, Smiley BL, Mills J, Vandenburgh HH: Mechanical stimulation improves tissue-engineered human skeletal muscle. Am.J.Physiol Cell Physiol 2002, 283: C1557-C1565.

[75] Pedrotty DM, Koh J, Davis BH, Taylor DA, Wolf P, Niklason LE: Engineering skeletal myoblasts: roles of three-dimensional culture and electrical stimulation. Am.J.Physiol.Heart Circ.Physiol. 2005, 288: H1620-H1626.
[76] Tanaka Y, Tsutsumi A, Crowe DM, Tajima S, Morrison WA: Generation of an autologous tissue (matrix) flap by combining an arteriovenous shunt loop with artificial skin in rats: preliminary report. Br.J.Plast.Surg. 2000, 53: 51-57.
[77] Mian R, Morrison WA, Hurley JV, Penington AJ, Romeo R, Tanaka Y, Knight KR: Formation of new tissue from an arteriovenous loop in the absence of added extracellular matrix. Tissue Eng. 2000, 6: 595-603.
[78] Shimizu T, Sekine H, Yang J, Isoi Y, Yamato M, Kikuchi A, Kobayashi E, Okano T: Polysurgery of cell sheet grafts overcomes diffusion limits to produce thick, vascularized myocardial tissues. FASEB J. 2006, 20: 708-710.
[79] Shimizu T, Sekine H, Isoi Y, Yamato M, Kikuchi A, Okano T: Long-term survival and growth of pulsatile myocardial tissue grafts engineered by the layering of cardiomyocyte sheets. Tissue Eng. 2006, 12: 499-507.
[80] Yang J, Yamato M, Shimizu T, Sekine H, Ohashi K, Kanzaki M, Ohki T, Nishida K, Okano T: Reconstruction of functional tissues with cell sheet engineering. Biomaterials 2007, 28: 5033-5043.
[81] Kubo H, Shimizu T, Yamato M, Fujimoto T, Okano T: Creation of myocardial tubes using cardiomyocyte sheets and an in vitro cell sheet-wrapping device. Biomaterials 2007, 28: 3508-3516.
[82] Ten Broek RW, Grefte S, Von den Hoff JW: Regulatory factors and cell populations involved in skeletal muscle regeneration. J.Cell.Physiol. 2010, 224: 7-16.
[83] Sacco A, Doyonnas R, Kraft P, Vitorovic S, Blau HM: Self-renewal and expansion of single transplanted muscle stem cells. Nature 2008, 456: 502-506.
[84] Buckingham M, Montarras D: Skeletal muscle stem cells. Curr.Opin.Genet.Dev. 2008.
[85] Le Grand F, Rudnicki MA: Skeletal muscle satellite cells and adult myogenesis. Curr.Opin.Cell Biol. 2007, 19: 628-633.
[86] Zammit P, Beauchamp J: The skeletal muscle satellite cell: stem cell or son of stem cell?. Differentiation 2001, 68: 193-204.
[87] Fukada S, Yamaguchi M, Kokubo H, Ogawa R, Uezumi A, Yoneda T, Matev MM, Motohashi N, Ito T, Zolkiewska A, et al.: Hesr1 and Hesr3 are essential to generate undifferentiated quiescent satellite cells and to maintain satellite cell numbers. Development 2011, 138: 4609-4619.
[88] Fukada S, Uezumi A, Ikemoto M, Masuda S, Segawa M, Tanimura N, Yamamoto H, Miyagoe-Suzuki Y, Takeda S: Molecular signature of quiescent satellite cells in adult skeletal muscle. Stem Cells 2007, 25: 2448-2459.
[89] Carnac G, Fajas L, L'honore A, Sardet C, Lamb NJ, Fernandez A: The retinoblastoma-like protein p130 is involved in the determination of reserve cells in differentiating myoblasts. Curr.Biol. 2000, 10: 543-546.
[90] Relaix F, Marcelle C: Muscle stem cells. Curr.Opin.Cell Biol. 2009, 21: 748-753.
[91] Cosgrove BD, Sacco A, Gilbert PM, Blau HM: A home away from home: challenges and opportunities in engineering in vitro muscle satellite cell niches. Differentiation 2009, 78: 185-194.
[92] McCarthy JJ: MicroRNA-206: the skeletal muscle-specific myomiR. Biochim.Biophys.Acta 2008, 1779: 682-691.
[93] Sousa-Victor P, Munoz-Canoves P, Perdiguero E: Regulation of skeletal muscle stem cells through epigenetic mechanisms. Toxicol.Mech.Methods 2011, 21: 334-342.
[94] Guller I, Russell AP: MicroRNAs in skeletal muscle: their role and regulation in development, disease and function. J.Physiol. 2010, 588: 4075-4087.
[95] Crist CG, Buckingham M: microRNAs gain magnitude in muscle. Cell.Cycle 2009, 8: 3627-3628.
[96] Crist CG, Buckingham M: Megarole for microRNA in muscle disease. Cell.Metab. 2010, 12: 425-426.
[97] Chen JF, Callis TE, Wang DZ: microRNAs and muscle disorders. J.Cell.Sci. 2009, 122: 13-20.
[98] Callis TE, Deng Z, Chen JF, Wang DZ: Muscling through the microRNA world. Exp.Biol.Med.(Maywood) 2008, 233: 131-138.
[99] Naguibneva I, Polesskaya A, Ameyar-Zazoua M, Souidi M, Groisman R, Cuvellier S, Ait-Si-Ali S, Pritchard LL, Harel-Bellan A: Micro-RNAs and muscle differentiation. J.Soc.Biol. 2007, 201: 367-376.
[100] Chen JF, Mandel EM, Thomson JM, Wu Q, Callis TE, Hammond SM, Conlon FL, Wang DZ: The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nat.Genet. 2006, 38: 228-233.
[101] Deng Z, Chen JF, Wang DZ: Transgenic overexpression of miR-133a in skeletal muscle. BMC Musculoskelet.Disord. 2011, 12: 115.

[102] Chen JF, Tao Y, Li J, Deng Z, Yan Z, Xiao X, Wang DZ: microRNA-1 and microRNA-206 regulate skeletal muscle satellite cell proliferation and differentiation by repressing Pax7. J.Cell Biol. 2010, 190: 867-879.
[103] Hirai H, Verma M, Watanabe S, Tastad C, Asakura Y, Asakura A: MyoD regulates apoptosis of myoblasts through microRNA-mediated down-regulation of Pax3. J.Cell Biol. 2010, 191: 347-365.
[104] Dey BK, Gagan J, Dutta A: miR-206 and -486 induce myoblast differentiation by downregulating Pax7. Mol.Cell.Biol. 2011, 31: 203-214.
[105] Koning M, Werker P, Bank RA, Harmsen MC: MicroRNA-1 and microRNA-206 improve differentiation potential of human satellite cells: A novel approach for tissue engineering of skeletal muscle. Tissue Eng.Part A. 2012, 18:
[106] Naguibneva I, Ameyar-Zazoua M, Polesskaya A, Ait-Si-Ali S, Groisman R, Souidi M, Cuvellier S, Harel-Bellan A: The microRNA miR-181 targets the homeobox protein Hox-A11 during mammalian myoblast differentiation. Nat.Cell Biol. 2006, 8: 278-284.
[107] Sarkar S, Dey BK, Dutta A: MiR-322/424 and -503 are induced during muscle differentiation and promote cell cycle quiescence and differentiation by down-regulation of Cdc25A. Mol.Biol.Cell 2010, 21: 2138-2149.
[108] Sun Q, Zhang Y, Yang G, Chen X, Zhang Y, Cao G, Wang J, Sun Y, Zhang P, Fan M, et al.: Transforming growth factor-beta-regulated miR-24 promotes skeletal muscle differentiation. Nucleic Acids Res. 2008, 36: 2690-2699.
[109] Crist CG, Montarras D, Pallafacchina G, Rocancourt D, Cumano A, Conway SJ, Buckingham M: Muscle stem cell behavior is modified by microRNA-27 regulation of Pax3 expression. Proc.Natl.Acad.Sci.U.S.A. 2009, 106: 13383-13387.
[110] Winbanks CE, Wang B, Beyer C, Koh P, White L, Kantharidis P, Gregorevic P: TGF-beta regulates miR-206 and miR-29 to control myogenic differentiation through regulation of HDAC4. J.Biol.Chem. 2011, 286: 13805-13814.
[111] Wang XH, Hu Z, Klein JD, Zhang L, Fang F, Mitch WE: Decreased miR-29 suppresses myogenesis in CKD. J.Am.Soc.Nephrol. 2011, 22: 2068-2076.
[112] Koning M, Werker PM, van Luyn MJ, Harmsen MC: Hypoxia promotes proliferation of human myogenic satellite cells: a potential benefactor in tissue engineering of skeletal muscle. Tissue Eng.Part A. 2011, 17: 1747-1758.

[113] Li XL, Andersen JB, Ezelle HJ, Wilson GM, Hassel BA: Post-transcriptional regulation of RNase-L expression is mediated by the 3'-untranslated region of its mRNA. J.Biol.Chem. 2007, 282: 7950-7960.
[114] Bisbal C, Silhol M, Laubenthal H, Kaluza T, Carnac G, Milligan L, Le Roy F, Salehzada T: The 2'-5' oligoadenylate/RNase L/RNase L inhibitor pathway regulates both MyoD mRNA stability and muscle cell differentiation. Mol.Cell.Biol. 2000, 20: 4959-4969.
[115] Bisbal C: RNase L: effector nuclease of an activatable RNA degradation system in mammals. Prog.Mol. Subcell.Biol. 1997, 18: 19-34.
[116] Rudnicki MA, Le Grand F, McKinnell I, Kuang S: The molecular regulation of muscle stem cell function. Cold Spring Harb.Symp.Quant.Biol. 2008, 73: 323-331.
[117] Kuang S, Gillespie MA, Rudnicki MA: Niche regulation of muscle satellite cell self-renewal and differentiation. Cell Stem Cell 2008, 2: 22-31.
[118] Brett JO, Renault VM, Rafalski VA, Webb AE, Brunet A: The microRNA cluster miR-106b~25 regulates adult neural stem/progenitor cell proliferation and neuronal differentiation. Aging (Albany NY) 2011, 3: 108-124.
[119] Anton R, Chatterjee SS, Simundza J, Cowin P, Dasgupta R: A systematic screen for micro-RNAs regulating the canonical Wnt pathway. PLoS One 2011, 6: e26257.
[120] Zhu H, Fan GC: Role of microRNAs in the reperfused myocardium towards post-infarct remodelling. Cardiovasc.Res. 2011.
[121] Salehzada T, Cambier L, Vu Thi N, Manchon L, Regnier L, Bisbal C: Endoribonuclease L (RNase L) regulates the myogenic and adipogenic potential of myogenic cells. PLoS One 2009, 4: e7563.
[122] Le Roy F, Silhol M, Salehzada T, Bisbal C: Regulation of mitochondrial mRNA stability by RNase L is translation-dependent and controls IFNalpha-induced apoptosis. Cell Death Differ. 2007, 14: 1406-1413.
[123] Andersen JB, Li XL, Judge CS, Zhou A, Jha BK, Shelby S, Zhou L, Silverman RH, Hassel BA: Role of 2-5A-dependent RNase-L in senescence and longevity. Oncogene 2007, 26: 3081-3088.
[124] Andersen JB, Mazan-Mamczarz K, Zhan M, Gorospe M, Hassel BA: Ribosomal protein mRNAs are primary targets of regulation in RNase-L-induced senescence. RNA Biol. 2009, 6: 305-315.
[125] Castelli JC, Hassel BA, Maran A, Paranjape J, Hewitt JA, Li XL, Hsu YT, Silverman RH, Youle RJ: The role

of 2'-5' oligoadenylate-activated ribonuclease L in apoptosis. Cell Death Differ. 1998, 5: 313-320.
[126] Zhou A, Paranjape JM, Hassel BA, Nie H, Shah S, Galinski B, Silverman RH: Impact of RNase L overexpression on viral and cellular growth and death. J.Interferon Cytokine Res. 1998, 18: 953-961.
[127] Ma J, Flemr M, Stein P, Berninger P, Malik R, Zavolan M, Svoboda P, Schultz RM: MicroRNA activity is suppressed in mouse oocytes. Curr.Biol. 2010, 20: 265-270.
[128] Suh N, Baehner L, Moltzahn F, Melton C, Shenoy A, Chen J, Blelloch R: MicroRNA function is globally suppressed in mouse oocytes and early embryos. Curr.Biol. 2010, 20: 271-277.
[129] Wu H, Neilson JR, Kumar P, Manocha M, Shankar P, Sharp PA, Manjunath N: miRNA profiling of naive, effector and memory CD8 T cells. PLoS One 2007, 2: e1020.
[130] Koning M, Harmsen MC, van Luyn MJ, Werker PM: Current opportunities and challenges in skeletal muscle tissue engineering. J Tissue Eng Regen.Med. 2009, 3: 407-415.
[131] Carosio S, Berardinelli MG, Aucello M, Musaro A: Impact of ageing on muscle cell regeneration. Ageing Res.Rev. 2011, 10: 35-42.
[132] Day K, Shefer G, Shearer A, Yablonka-Reuveni Z: The depletion of skeletal muscle satellite cells with age is concomitant with reduced capacity of single progenitors to produce reserve progeny. Dev.Biol. 2010, 340: 330-343.
[133] Corbu A, Scaramozza A, Badiali-DeGiorgi L, Tarantino L, Papa V, Rinaldi R, D'Alessandro R, Zavatta M, Laus M, Lattanzi G, Cenacchi G: Satellite cell characterization from aging human muscle. Neurol.Res. 2010, 32: 63-72.
[134] Gopinath SD, Rando TA: Stem cell review series: aging of the skeletal muscle stem cell niche. Aging Cell. 2008, 7: 590-598.
[135] Biressi S, Rando TA: Heterogeneity in the muscle satellite cell population. Semin.Cell Dev.Biol. 2010, 21: 845-854.
[136] Kallestad KM, McLoon LK: Defining the heterogeneity of skeletal muscle-derived side and main population cells isolated immediately ex vivo. J.Cell.Physiol. 2010, 222: 676-684.
[137] Rao PK, Kumar RM, Farkhondeh M, Baskerville S, Lodish HF: Myogenic factors that regulate expression of muscle-specific microRNAs. Proc.Natl.Acad.Sci.U.S.A. 2006, 103: 8721-8726.
[138] Yuasa K, Hagiwara Y, Ando M, Nakamura A, Takeda S, Hijikata T: MicroRNA-206 is highly expressed in newly formed muscle fibers: implications regarding potential for muscle regeneration and maturation in muscular dystrophy. Cell Struct.Funct. 2008, 33: 163-169.
[139] Zammit PS: All muscle satellite cells are equal, but are some more equal than others?. J.Cell.Sci. 2008, 121: 2975-2982.
[140] Peault B, Rudnicki M, Torrente Y, Cossu G, Tremblay JP, Partridge T, Gussoni E, Kunkel LM, Huard J: Stem and progenitor cells in skeletal muscle development, maintenance, and therapy. Mol.Ther. 2007, 15: 867-877.
[141] Kuang S, Rudnicki MA: The emerging biology of satellite cells and their therapeutic potential. Trends Mol. Med. 2008, 14: 82-91.
[142] Olguin HC, Olwin BB: Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal. Dev.Biol. 2004, 275: 375-388.
[143] Rhim C, Cheng CS, Kraus WE, Truskey GA: Effect of microRNA modulation on bioartificial muscle function. Tissue Eng.Part A. 2010, 16: 3589-3597.
[144] van der Schaft DW, van Spreeuwel AC, van Assen HC, Baaijens FP: Mechanoregulation of vascularization in aligned tissue-engineered muscle: a role for vascular endothelial growth factor. Tissue Eng.Part A. 2011, 17: 2857-2865.
[145] Charge SB, Rudnicki MA: Cellular and molecular regulation of muscle regeneration. Physiol.Rev. 2004, 84: 209-238.
[146] Zammit PS, Relaix F, Nagata Y, Ruiz AP, Collins CA, Partridge TA, Beauchamp JR: Pax7 and myogenic progression in skeletal muscle satellite cells. J.Cell.Sci. 2006, 119: 1824-1832.
[147] Boldrin L, Muntoni F, Morgan JE: Are human and mouse satellite cells really the same?. J.Histochem. Cytochem. 2010, 58: 941-955.
[148] Langelaan ML, Boonen KJ, Rosaria-Chak KY, van der Schaft DW, Post MJ, Baaijens FP: Advanced maturation by electrical stimulation: Differences in response between C2C12 and primary muscle progenitor cells. J.Tissue Eng.Regen.Med. 2010.
[149] Collins CA, Olsen I, Zammit PS, Heslop L, Petrie A, Partridge TA, Morgan JE: Stem cell function, self-

renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. Cell 2005, 122: 289-301.
[150] Ono Y, Boldrin L, Knopp P, Morgan JE, Zammit PS: Muscle satellite cells are a functionally heterogeneous population in both somite-derived and branchiomeric muscles. Dev.Biol. 2010, 337: 29-41.
[151] Yoshida N, Yoshida S, Koishi K, Masuda K, Nabeshima Y: Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates 'reserve cells'. J.Cell.Sci. 1998, 111 (Pt 6): 769-779.
[152] Manzano R, Toivonen JM, Calvo AC, Miana-Mena FJ, Zaragoza P, Munoz MJ, Montarras D, Osta R: Sex, fiber-type and age dependent in vitro proliferation of mouse muscle satellite cells. J.Cell.Biochem. 2011.
[153] Terng SC, Rijnders W, Kon M, Werker PM: [Custom-made gold plate prosthesis in the upper eyelid in patients with facial paralysis: improved eyelid closure with less effects on other eye complaints]. Ned.Tijdschr. Geneeskd. 2000, 144: 800-804.
[154] Liao H, Zhou GQ: Development and progress of engineering of skeletal muscle tissue. Tissue Eng.Part B.Rev. 2009, 15: 319-331.
[155] Bouchentouf M, Benabdallah BF, Bigey P, Yau TM, Scherman D, Tremblay JP: Vascular endothelial growth factor reduced hypoxia-induced death of human myoblasts and improved their engraftment in mouse muscles. Gene Ther. 2008, 15: 404-414.
[156] Hayot M, Rodriguez J, Vernus B, Carnac G, Jean E, Allen D, Goret L, Obert P, Candau R, Bonnieu A: Myostatin up-regulation is associated with the skeletal muscle response to hypoxic stimuli. Mol.Cell.Endocrinol. 2010.
[157] Hu X, Yu SP, Fraser JL, Lu Z, Ogle ME, Wang JA, Wei L: Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function via enhanced survival of implanted cells and angiogenesis. J.Thorac.Cardiovasc.Surg. 2008, 135: 799-808.
[158] Kook SH, Son YO, Lee KY, Lee HJ, Chung WT, Choi KC, Lee JC: Hypoxia affects positively the proliferation of bovine satellite cells and their myogenic differentiation through up-regulation of MyoD. Cell Biol.Int. 2008, 32: 871-878.
[159] Menasche P, Alfieri O, Janssens S, McKenna W, Reichenspurner H, Trinquart L, Vilquin JT, Marolleau JP, Seymour B, Larghero J, et al.: The Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial: first randomized placebo-controlled study of myoblast transplantation. Circulation 2008, 117: 1189-1200.
[160] Csete M, Walikonis J, Slawny N, Wei Y, Korsnes S, Doyle JC, Wold B: Oxygen-mediated regulation of skeletal muscle satellite cell proliferation and adipogenesis in culture. J.Cell.Physiol. 2001, 189: 189-196.
[161] Chakravarthy MV, Spangenburg EE, Booth FW: Culture in low levels of oxygen enhances in vitro proliferation potential of satellite cells from old skeletal muscles. Cell Mol.Life Sci. 2001, 58: 1150-1158.
[162] McLoon LK, Wirtschafter JD: N-CAM is expressed in mature extraocular muscles in a pattern conserved among three species. Invest.Ophthalmol.Vis.Sci. 1996, 37: 318-327.
[163] Yun Z, Lin Q, Giaccia AJ: Adaptive myogenesis under hypoxia. Mol.Cell.Biol. 2005, 25: 3040-3055.
[164] Di Carlo A, De Mori R, Martelli F, Pompilio G, Capogrossi MC, Germani A: Hypoxia inhibits myogenic differentiation through accelerated MyoD degradation. J.Biol.Chem. 2004, 279: 16332-16338.
[165] Rossi CA, Pozzobon M, Ditadi A, Archacka K, Gastaldello A, Sanna M, Franzin C, Malerba A, Milan G, Cananzi M, et al.: Clonal characterization of rat muscle satellite cells: proliferation, metabolism and differentiation define an intrinsic heterogeneity. PLoS.ONE. 2010, 5: e8523.
[166] Friday BB, Pavlath GK: A calcineurin- and NFAT-dependent pathway regulates Myf5 gene expression in skeletal muscle reserve cells. J.Cell.Sci. 2001, 114: 303-310.
[167] Cao Y, Zhao Z, Gruszczynska-Biegala J, Zolkiewska A: Role of metalloprotease disintegrin ADAM12 in determination of quiescent reserve cells during myogenic differentiation in vitro. Mol.Cell.Biol. 2003, 23: 6725-6738.
[168] Kitzmann M, Bonnieu A, Duret C, Vernus B, Barro M, Laoudj-Chenivesse D, Verdi JM, Carnac G: Inhibition of Notch signaling induces myotube hypertrophy by recruiting a subpopulation of reserve cells. J.Cell. Physiol. 2006, 208: 538-548.
[169] Jacquemin V, Butler-Browne GS, Furling D, Mouly V: IL-13 mediates the recruitment of reserve cells for fusion during IGF-1-induced hypertrophy of human myotubes. J.Cell.Sci. 2007, 120: 670-681.
[170] Rapizzi E, Donati C, Cencetti F, Nincheri P, Bruni P: Sphingosine 1-phosphate differentially regulates proliferation of C2C12 reserve cells and myoblasts. Mol.Cell.Biochem. 2008, 314: 193-199.
[171] Stuelsatz P, Pouzoulet F, Lamarre Y, Dargelos E, Poussard S, Leibovitch S, Cottin P, Veschambre P: Down-regulation of MyoD by calpain 3 promotes generation of reserve cells in C2C12 myoblasts. J.Biol.Chem.

2010, 285: 12670-12683.
[172] Usas A, Huard J: Muscle-derived stem cells for tissue engineering and regenerative therapy. Biomaterials 2007, 28: 5401-5406.
[173] Rhoads RP, Johnson RM, Rathbone CR, Liu X, Temm-Grove C, Sheehan SM, Hoying JB, Allen RE: Satellite cell-mediated angiogenesis in vitro coincides with a functional hypoxia-inducible factor pathway. Am.J Physiol Cell Physiol 2009, 296: C1321-C1328.
[174] Verseijden F, Posthumus-van Sluijs SJ, Pavljasevic P, Hofer SO, van Osch GJ, Farrell E: Adult human bone marrow- and adipose tissue-derived stromal cells support the formation of prevascular-like structures from endothelial cells in vitro. Tissue Eng.Part A. 2010, 16: 101-114.
[175] Saleh FA, Whyte M, Genever PG: Effects of endothelial cells on human mesenchymal stem cell activity in a three-dimensional in vitro model. Eur.Cell.Mater. 2011, 22: 242-257.
[176] Garzoni LR, Rossi MI, de Barros AP, Guarani V, Keramidas M, Balottin LB, Adesse D, Takiya CM, Manso PP, Otazu IB, et al.: Dissecting coronary angiogenesis: 3D co-culture of cardiomyocytes with endothelial or mesenchymal cells. Exp.Cell Res. 2009, 315: 3406-3418.
[177] Christov C, Chretien F, bou-Khalil R, Bassez G, Vallet G, Authier FJ, Bassaglia Y, Shinin V, Tajbakhsh S, Chazaud B, Gherardi RK: Muscle satellite cells and endothelial cells: close neighbors and privileged partners. Mol.Biol.Cell 2007, 18: 1397-1409.
[178] Lesman A, Koffler J, Atlas R, Blinder YJ, Kam Z, Levenberg S: Engineering vessel-like networks within multicellular fibrin-based constructs. Biomaterials 2011, 32: 7856-7869.
[179] Koffler J, Kaufman-Francis K, Yulia S, Dana E, Daria AP, Landesberg A, Levenberg S: Improved vascular organization enhances functional integration of engineered skeletal muscle grafts. Proc.Natl.Acad.Sci.U.S.A. 2011, 108: 14789-14794.
[180] Verseijden F, Posthumus-van Sluijs SJ, Farrell E, van Neck JW, Hovius SE, Hofer SO, van Osch GJ: Pre-vascular structures promote vascularization in engineered human adipose tissue constructs upon implantation. Cell Transplant. 2010, 19: 1007-1020.
[181] Tulloch NL, Muskheli V, Razumova MV, Korte FS, Regnier M, Hauch KD, Pabon L, Reinecke H, Murry CE: Growth of engineered human myocardium with mechanical loading and vascular coculture. Circ.Res. 2011, 109: 47-59.
[182] Langelaan ML, Boonen KJ, Rosaria-Chak KY, van der Schaft DW, Post MJ, Baaijens FP: Advanced maturation by electrical stimulation: Differences in response between C2C12 and primary muscle progenitor cells. J.Tissue Eng.Regen.Med. 2011, 5: 529-539.
[183] Burgess WH, Mehlman T, Friesel R, Johnson WV, Maciag T: Multiple forms of endothelial cell growth factor. Rapid isolation and biological and chemical characterization. J.Biol.Chem. 1985, 260: 11389-11392.
[184] Popa ER, Harmsen MC, Tio RA, van der Strate BW, Brouwer LA, Schipper M, Koerts J, De Jongste MJ, Hazenberg A, Hendriks M, van Luyn MJ: Circulating CD34+ progenitor cells modulate host angiogenesis and inflammation in vivo. J.Mol.Cell.Cardiol. 2006, 41: 86-96.
[185] Bryan BA, Walshe TE, Mitchell DC, Havumaki JS, Saint-Geniez M, Maharaj AS, Maldonado AE, D'Amore PA: Coordinated vascular endothelial growth factor expression and signaling during skeletal myogenic differentiation. Mol.Biol.Cell 2008, 19: 994-1006.
[186] Germani A, Di CA, Mangoni A, Straino S, Giacinti C, Turrini P, Biglioli P, Capogrossi MC: Vascular endothelial growth factor modulates skeletal myoblast function. Am.J.Pathol. 2003, 163: 1417-1428.
[187] Borselli C, Storrie H, esch-Lee F, Shvartsman D, Cezar C, Lichtman JW, Vandenburgh HH, Mooney DJ: Functional muscle regeneration with combined delivery of angiogenesis and myogenesis factors. Proc.Natl. Acad.Sci.U.S.A. 2009.
[188] Borselli C, Cezar CA, Shvartsman D, Vandenburgh HH, Mooney DJ: The role of multifunctional delivery scaffold in the ability of cultured myoblasts to promote muscle regeneration. Biomaterials 2011, 32: 8905-8914.
[189] Evensen L, Micklem DR, Blois A, Berge SV, Aarsaether N, Littlewood-Evans A, Wood J, Lorens JB: Mural cell associated VEGF is required for organotypic vessel formation. PLoS.ONE. 2009, 4: e5798.
[190] Gaengel K, Genove G, Armulik A, Betsholtz C: Endothelial-mural cell signaling in vascular development and angiogenesis. Arterioscler.Thromb.Vasc.Biol. 2009, 29: 630-638.
[191] Takahashi A, Kureishi Y, Yang J, Luo Z, Guo K, Mukhopadhyay D, Ivashchenko Y, Branellec D, Walsh K: Myogenic Akt signaling regulates blood vessel recruitment during myofiber growth. Mol.Cell.Biol. 2002, 22: 4803-4814.
[192] Stal P, Eriksson PO, Eriksson A, Thornell LE: Enzyme-histochemical differences in fibre-type between the

human major and minor zygomatic and the first dorsal interosseus muscles. Arch.Oral Biol. 1987, 32: 833-841.
[193] Happak W, Burggasser G, Gruber H: Histochemical characteristics of human mimic muscles. J.Neurol. Sci. 1988, 83: 25-35.
[194] Rosenblatt JD, Parry DJ, Partridge TA: Phenotype of adult mouse muscle myoblasts reflects their fiber type of origin. Differentiation 1996, 60: 39-45.
[195] Gaetani R, Doevendans PA, Metz CH, Alblas J, Messina E, Giacomello A, Sluijter JP: Cardiac tissue engineering using tissue printing technology and human cardiac progenitor cells. Biomaterials 2012, 33: 1782-1790.
[196] Peltola SM, Melchels FP, Grijpma DW, Kellomaki M: A review of rapid prototyping techniques for tissue engineering purposes. Ann.Med. 2008, 40: 268-280.
[197] Moore AM, Borschel GH, Santosa KA, Flagg ER, Tong AY, Kasukurthi R, Newton P, Yan Y, Hunter DA, Johnson PJ, Mackinnon SE: A transgenic rat expressing green fluorescent protein (GFP) in peripheral nerves provides a new hindlimb model for the study of nerve injury and regeneration. J.Neurosci.Methods 2011, 204: 19-27.
[198] Frey M, Giovanoli P, Meuli-Simmen C: The qualification of different free muscle transplants to reconstruct mimic function: an experimental study in rabbits. Plast.Reconstr.Surg. 1998, 101: 1774-1783.

NEDERLANDSE SAMENVATTING

Verlamming van het aangezicht is behalve een fysieke, ook een sociaal invaliderende aandoening. In een wereld waarin miljoenen besteed worden aan het uiterlijk, leidt een scheef gezicht bij een niet gering percentage van de patiënten tot ernstige psychische stress en zelfs depressiviteit. Geschat wordt dat er jaarlijks zo'n 4.000 personen in Nederland een aangezichtsverlamming krijgen. Van deze patiënten, houden er zo'n 1.200 klachten en verschijnselen van de aangezichtsverlamming. Een groot deel van hen is met succes te behandelen. Er is een grote vooruitgang op het gebied van de reconstructieve chirurgie geboekt, en ook conservatieve bahandelstrategiën met bijvoorbeeld een mimetherapeut worden steeds verder ontwikkeld. Echter blijven er patiënten met deels onbehandelbare klachten zoals synkinesieën en spasmen, lagophthalmus en asymmetrie van het aangezicht. Voor deze groep patiënten moeten nieuwe, innovatieve behandelstrategieën ontwikkeld worden. Regenerative geneeskunde, en tissue engineering in het bijzonder, geeft een optie voor een potentiële nieuwe behandelstrategie.

Doel van dit proefschrift was om tissue engineering van de menselijke skeletspier te verbeteren door het aanbieden van nieuwe fundamentele inzichten en tools.
Daarvoor hebben we in hoofdstuk 2 de stand van zaken op het gebied van tissue engineering van skelet spieren in kaart gebracht. Daaruit kwam naar voren dat een juiste voorloper cel en scaffold (dat is een soort steiger of kapstok) essentieel zijn in tissue engineering. Wij vonden dat, uit verschillende voorloper cellen zoals beenmerg stamcellen, vet stamcellen en de muizen cellijn C2C12, menselijke satelliet cellen het meest geschikt zijn als bron voor tissue engineering van skeletspieren. Het gebruik van een 3D biologisch afbreekbare scaffold op basis van extracellulaire matrix, zoals fibrine of collageen, is geschikt om structurele steun te bieden aan cellen en bovendien de proliferatie en differentiatie van satelliet cellen te reguleren. Vervolgens is voor de overleving van getissue engineerde spieren een connectie met vasculaire stelsel vereist. Verschillende methoden om dit te bereiken zijn: 1) een vasculaire pedicle, 2) een geprevasculariseerde scaffold, 3) ingroei van de vaten vanuit de ontvanger, bijvoorbeeld een methode waar lagen cellen gestapeld worden. Een andere voorwaarde voor het engineeren van functionele skeletspieren is een adequate verbinding met het zenuwstelsel. Dit kan bereikt worden door het toepassen van elektrische, chemotrofische of mechanische stimulatie tijdens engineering.
Echter, in het kader van engineering van autolog menselijk skeletspier, is er behoefte aan fundamentele kennis betreffende menselijke satelliet cellen. Daarvoor werd de differentiatie capaciteit van gekloonde menselijke satelliet cellen bestudeerd in hoofd-

stuk 3. We zagen dat menselijke satelliet cellen tijdens differentiatie *in vitro*, hetzij myotubes, dat zijn onvolwassen spiervezels, hetzij nieuwe quiescent satelliet cellen vormen. Vervolgens bestudeerden we het reguleringsmechanisme tijdens de differentiatie op post-transcriptioneel niveau. Ze zagen dat over het algemeen microRNA's afnemen in quiescent satelliet cellen. Verder bevestigen we dat microRNA-1 en microRNA-206 sterk verhoogde zijn in myotubes.
De therapeutische toepassing van deze vindingen in tissue engineering is in hoofdtuk 4 aan de orde. Omdat gedurende ons leven de regeneratieve capaciteiten van onze satelliet cellen achteruit gaat, is verbetering van differentiatie vooral voor oudere patienten belangrijk. De microRNA's die mede verantwoordelijk zijn voor het bevorderen van spiervezelformatie hebben we toegepast op satelliet cellen in een *in vitro* 3D model. Met deze methode worden spierconstructen gemaakt van humane satelliet cellen en collageen type I. Met de manipulatie van de satelliet cellen door microRNA's kunnen we inderdaad de spiervezel formatie verbeteren.

Een andere belangrijke fundamentele kwestie is dat satelliet cellen op het moment van implantatie in een patiënt, in een zuurstofarm milieu terecht zullen komen. Hypoxie heeft een sterke invloed op de overleving, proliferatie en differentiatie van humane satelliet cellen. Daarom hebben we in hoofdstuk 5 onderzocht hoe satelliet cellen reageren op een lage zuurstof spanning, en wat er gebeurt op het moment dat satelliet cellen in een zuurstofarm milieu differentieren. We zagen dat humane satelliet cellen zeer goed bestand zijn tegen een lage zuurstofspanning. Ze worden kortstondig aangespoord om te prolifereren, en verliezen bovendien niets van hun myogene capaciteiten. Dit maakt humane satelliet cellen zeer geschikt voor tissue engineering van de skeletspieren voor klinische toepassingen.
Tenslotte worden in hoofdstuk 6 de differentiatie en maturatie van gekloonde menselijke satelliet cellen onderzocht in een 3D collageen I spier construct dat subcutaan in naakt ratten is geïmplanteerd. Bovendien, met betrekking tot de *in vivo* vascularisatie, hebben we ook het effect onderzocht van *in vitro* preconditionering van deze spier constructen met humane navelstreng endotheelcellen (HUVEC). We zien dat gekloonde menselijke satelliet cellen volledig matureren *in vivo* en differentiëren in spiervezels en quiescent satelliet cellen. Deze gematureerde spiervezels werden goed gevasculariseerd door vaten van de naakte rat. Dit specifiek als gevolg van de humande satelliet cellen, te concluderen uit het feit dat de collageen type I gels zelf geen vascularisatie induceerden. De spiervezel maturatie en ook de vascularisatie weren beide niet veranderd door het preconditioneren met HUVEC.

Kortom, de nieuwe inzichten en handvaten die we hebben besproken in dit proefschrift vormen een stevige basis voor tissue engineering van de menselijke skeletspier van menselijke satelliet cellen. We hebben aangetoond dat satelliet cellen en myotubes in een collageen type I construct subcutaan differentiëren en matureren tot goed doorbloedde, volwassen spiervezels en quiescent satelliet cellen. Verder hebben we aangetoond dat gekloonde menselijke satelliet cellen gemoduleerd kunnen worden door microRNA's. Dit biedt, vooral bij oudere patiënten, mogelijkheden om het engineeren van skelet spier met behulp van autologe satelliet cellen te verbeteren.

DANKWOORD

Hoe vaak krijg je nou de kans om de mensen die je waardeert te kunnen bedanken ‘in print’? En kansen moet je grijpen, want ik ben jullie dankbaar, zonder jullie had ik dit proefschrift niet geschreven.

Mijn eerste promotor prof. dr. P.M.N. Werker. Beste Paul, jij hebt altijd the big picture voor ogen waardoor dit proefschrift de klinische inslag heeft gekregen die we vanaf de start voor ogen hadden. Dankbaar ben ik ook voor je vertrouwen en het geduld dat je hebt gehad. Dat zijn essentiële ingrediënten voor een beginnend onderzoeker. Ik kijk uit naar de volgende fase waarin je mijn opleider bent.

Mijn co-promotor, dr. M.C. Harmsen. Beste Marco, zonder jou had deze promotie niet de vorm gekregen die hij nu heeft. Met je bevlogenheid voor wetenschap weet je ook mij elke keer weer te enthousiasmeren. Dank voor alles wat je me geleerd hebt, van tevoren had ik dit niet gedacht. Jou wetenschaps-, onderwijs- en schrijfkunsten hebben mij tot onderzoeker gevormd.

Mijn tweede promotor, prof. dr. M.J.A. van Luyn. Beste Marja, in de slotfase hebben we spijkers met koppen geslagen. Dank voor je betrokkenheid bij het onderzoek, met name hoofdstuk 6 is er prachtig door geworden. En door je betrokkenheid bij mij als persoon ben ik een beter mens geworden. Trots als een pauw!

Dr. D.W.J. van der Schaft, beste Daisy, wat een geluk dat wij elkaar in Davos tegen het lijf zijn gelopen. Het heeft tot hoofdstuk 4 én hoofdstuk 6 geleid, een geslaagd congres dus! Van jou toepassings gerichte kijk op tissue engineering heb ik veel geleerd.

Prof. dr. R.A. Bank, beste Ruud, dank voor je kortstondige begeleiding. Het is ontzettend fijn dat je mij en mijn onderzoek hebt willen ‘adopteren’. Het heeft tot een mooi hoofdstuk 4 geleid en bij mij een interesse voor collageen en fibrose.

Natuurlijk wil in de leescommissie, Prof. dr. Berend van der Lei, Prof. dr. Mark H. Post en Prof. dr. Wout F.J. Feitz bedanken voor de uitvoer van de belangrijke taak om dit proefschrift te beoordelen.

Zonder collega’s op de werkvloer was het helemaal niets geworden, zo groen als gras begonnen aan dit project, nog nooit een pipet gezien. Maar jullie hebben mij wegwijsgemaakt, zowel praktisch als bij het interpreteren en uitwerken van data. De

ontelbare kopjes koffie dan wel biertjes met bijbehorende wetenschappelijke en vaak ook minder wetenschappelijke discussies hebben mij de afgelopen 4 jaar doorgeholpen. De AIO's Jan-Reinier Moonen, Sander van Putten (mijn lunch- en koffie maatje, ik denk inderdaad dat mensen productiever zijn als ze lekker kunnen klagen :-)), Ee Soo Lee, Ewa Przybyt, Nynke Hospers, Diana Ploeger, Jurjen Zandstra, Rutger Gjaltema, Marloes de Groote, Christian Huisman, Mojtaba Parvizi, Monika Maleszewska, Gazele Hajmousa en Nina Schweinfurth.
De analysten Josee Plantinga, Marja Brinker, Jasper Koerts, Peter Zwiers, Martin Schipper, Henk Moorlag, Anita Niemarkt, Linda Brouwer, Saskia de Rond, Inge Kazemier, Henriette Morselt, Jelleke Dokter, Henk Moes, Roelof-Jan van de Leij, Geert Mesander, Arjen Peters en Klaas Sjollema. Natuurlijk hoort Jochem Prop in dit dankwoord thuis die mij met zijn kennis en kunde door de Dec aanvragen heeft geloodst. De post-doc's Guido Krenning (dank voor de vele broodkruimels), Eliane Popa, Marike van Beuge en Patricia Dankers.
Ook studenten maken onmiskenbaar deel uit van het lab, en van hen begeleiden en onderwijzen heb ik veel geleerd, bedankt! Erik, dank voor de fanastische opmaat van al het werk dat volgde. Anne en Karlijn, voor jullie pionierwerk met de microRNA's en Jacqueline voor de hulp met het *in vivo* werk.

Ik heb het geluk gehad in mijn promotietijd ook bij de afdeling plastische chirugie te horen, en alle daaruit voortvloeiende gezelligheid. Het jaarlijkse skitripje, congressen, borrels, zeilen en kopjes koffie/alcohol helpen om af en toe het lab uit te komen. De plastisch chirurgen Berend van der Lei, Yassir Eltahir, Tim Middelberg, Mike Rüttermann, Thomas Wermter, Dagna Lichtendahl, Lars Lahoda (fijn om de passie voor wetenschap en congressen te delen), Wiebren van de Berg, Martin Stenekes, en zeker Coralien Broekhuizen en Irene Krabbe (dank voor jullie inzet bij het vorm geven aan mijn opleidingsplek).
De AIOS Jesse van Lieshout, Steven Klein, Steven Korteweg, Vick Sreetharan, Patrick Khoe, Marc Kioe-A-Sen, Marijn Huijing (de huizenjacht, vele biertjes met bitterballen en het fietsen waren allemaal top!), Mireille Bolster, Ellen Meerman, Johan Wijbenga (fijn dat je nu al mijn collega bent) en Rinze Zwanenburg (op naar alle cursussen die nog volgen). Ook Jacoline (altijd gezellig om even een praatje te maken), Elisabeth Sijbesma, Etty de Jong, Antoinette van Minnen en Bert Tebbes (fietsen en foto's, daarover kunnen we eindeloos kletsen). En de plastisch chirurgen en artsen uit Leeuwarden wil ik bedanken voor de gezelligheid door de jaren heen, met name dr. J.J. van der Biezen, Eddie Verschuur en Marije Smittenberg voor het fietsen en skiën.

De chirurgen en artsen uit het Martini Ziekenhuis, ik vind het heerlijk weer terug te zijn in de kliniek en met jullie de volgende uitdaging aan te gaan.

Mijn collega's van het SWOAHS, dank dat ik de komende jaren nog mag genieten van jullie goede gezelschap, goed eten en natuurlijk de wetenschap.

In de loop der jaren hebben velen anderen mijn pad gekruist met koffie, drankjes, onderzoek en natuurlijk de daarbij gepaard gaande gesprekken, een paar wil ik bij naam noemen: Niels Harlaar, Wendeline van Eede, Anton Breeuwsma, Nico van Bakelen, Renske Dikkers, Evert-Jan ten Dam, Rosanne Lanting en Sophie Post.

Mijn vrienden Danielle en Barry met de kids, fijn dat julle mijn vrienden zijn. Jurrijn en Monique, Bart en Roelfien, al sinds onze tijd bij Volonté geniet ik van onze activiteiten samen, dat er nog maar veel mogen volgen.

Mijn jaarclub Blixem, wat heb ik een geluk dat ik jullie mijn vriendinnen mag noemen! Mooie tijden, alweer het 2e lustrum en nóg steeds vinden we elk jaar wel een mogelijkheid om samen op vakantie te gaan, op naar het 3e! Ik zou wensen dat Groningen niet zover was, maar des te meer geniet ik van de etentjes, feestjes, het winkelen, fietsen en de weekendjes weg.

Corrie en Naud, Agniet en Koos, jullie aanwezigheid tijdens mijn jonge jaren hebben mij onmiskenbaar gevormd tot wie ik ben.

Mijn paranimfen Miriam en Guido, dolblij dat jullie hier naast mij staan. En ik ben ontzettend trots en vereerd ook jullie straks bij te mogen staan!
Lieve Miriam, mijn steun en toeverlaat, zowel op als buiten het lab. Je bent de meest gastvrije en mooie persoon die ik ken, ik maak altijd met veel plezier gebruik van de gezelligheid bij jou en Marten thuis. Onze zaterdagmiddagbiertjes zijn een essentieel ingredient om de week te bespreken en vol goede moed de nieuwe week het hoofd te kunnen bieden.
Beste Guido, mijn lot- en deelgenoot, de vele uurtjes op de golfbaan waren rust momenten die onontbeerlijk zijn tijdens onderzoek. Ook de etentjes en zeiltochtjes met Denise fungeren elke keer als een soort minivakantie. Ik hoop dat er nog vele activiteiten mogen volgen.

Benny en Cisca, het is fijn om zoveel warmte te krijgen in jullie gezin, ik ben blij dat ik er deel van uit mag maken. Jeffrey, mijn schoonbroer, ik denk dat jij het beste begreep wat ik deed, en hoe het eraan toe gaat.

Hans en Wendy, Marc en Ellen, Martijn en Famke en natuurlijk alle kids, fijn dat jullie deel uit maken van mijn familie, altijd goed om jullie te zien en spreken, met de kinderen te spelen of in de Porsche te cruisen.
Guido en Yvonne, Guido, ik ben ontzettend trots op jou en de manier waarop jij van het leven geniet, daar kan ik nog veel van leren. Yvonne, ik vind het heel fijn dat jij mijn schoonzusje bent.

Lieve mam en Henk. Mam, je bent mijn grote voorbeeld, de kracht waarmee jij in het leven staat vind ik bewonderingswaardig. De dagjes die we af en toe samen wat leuks doen zijn heel waardevol voor mij. Ook pap geniet vandaag met ons mee, hij is er altijd bij. Henk, fijn dat jij ons gezin weer compleet hebt gemaakt, ik heb het heel goed gehad in Apeldoorn. Ik floreer door jullie steun.

Lieve Kevin, je bent fantastisch! Jouw liefde, steun en begrip zijn essentieel voor mij, het is mijn drijvende kracht. Ik geniet met volle teugen van elke dag met jou, en ons leven samen.